DEVELOPMENT OF CANINE BIOMARKER BASED DETECTION AND QUANTIFICATION ASSAYS FOR DETERMINING FOOD ADULTERATION AND HALAL AUTHENTICATION

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INSTITUTE OF GRADUATE STUDIES
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ABSTRACT

The authentication of food product is an important issue to safeguard consumer rights, religious belief, and health and fair-trade economy. Proper labeling of food products allows consumers to make a well-informed purchase decision, commensurations his religious faith, health requirement and of course his personal budget. “Halal” logo on food products reflects both the ingredients and processing steps of the products confirm the Shariah Principles of Islam for hygienic foods. Since halal foods serve the health and religious requirements, their appeal is huge even to the non-Muslim customers. The huge turnover (US$700 billion/ annum) and expanding demands of halal food products testify their widespread popularity throughout the world. The specialized processing, stringent supply chain and safety standard of halal foods have made them costlier to their non-halal counterparts and consequently halal branded products have been targeted for fraud labeling for years.

Dog meat consumption is forbidden in Islam and it has been a less discussed issue for fraudulent mixing in halal foods. However, report has been published for the consumption of dog meats by Vietnamese and Myanmarian workers in Malaysia. Dog meat consumptions are also common in South Korea, China and Vietnam. Additionally, the widespread availability of stray dogs without any offered prices in many countries has made it as a highly potential adulterant in halal foods.

In literature, five polymerase chain reactions (PCR) assays have been proposed for the detection of dog meats. However, most of the documented assays have targeted longer DNA fragment (≥213 bp) and none of them have tested under complex food matrices. Longer DNA fragments frequently break down during food processing, causing false negative detection in the final assay. To overcome the potential limitations of the existing assays, we have developed short DNA fragment based conventional PCR, PCR-RFLP and

real-time PCR assays targeting a 100-bp site of mitochondrial cytochrome b gene. The specificity of the assays were tested using DNA extracted from dog and common meat providing land, aquatic, bird and plant species. The assay stability was tested under different food processing conditions, including boiling, autoclaving and oven heating under pure, admixed and commercial food matrices and was found to be highly stable. The developed conventional PCR assay successfully detected 0.1% to 0.2% (w/w) canine meat from admixed and commercial samples, reflecting its stability and sensitivity under complex matrices. PCR products were authenticated by digesting the product with *AluI* restriction enzyme which generated 51-, 30- and 19-bp fragments. The digested fragments were successfully separated using Experion Bioanalyzer kit. The sensitivity of the PCR-RFLP assay was 0.0001-ng canine DNA under pure and 0.01% (w/w) canine meat spiked in chicken and beef burger formulations. Finally, a TaqMan probe real-time PCR assay was developed and it was found highly stable and sensitive both under pure and complex matrices. The developed real time-PCR assay successfully detected 0.01% (w/w, 0.002 ng DNA) canine meat spiked in commercial chicken nuggets. A total of six halal branded food products obtained from different Malaysian outlets were screen and no canine adulteration was detected.

ABSTRAK

Keaslian sesuatu produk makanan merupakan isu yang amat penting demi menjaga hak pengguna, kepercayaan agama, kesihatan awam dan perkembangan ekonomi. Bungkusan haruslah dilabel dengan tepat kerana ianya adalah amat penting bagi pengguna supaya mereka untuk membuat keputusan dalam membeli. Logo "Halal" pada setiap produk makanan adalah sebagai garis penanda bahawa produk tersebut mengikut prinsip-prinsip Syariah Islam dan bahan-bahannya selari dengan ajaran Islam.Memandangkan,makanan halal memenuhi kehendak dari sudut agama dan kesihatan,permintaan terhadapnya bertambah termasuk dari pengguna bukan islam.Pulangan yang melebihi AS $700 bilion dalam setahun dan permintaan yang semakin tinggi membuatkan ia kian popular di serata dunia. Pemprosesan khusus, bekalan bahan yang terhad dan piawaian keselamatan yang ada pada makanan halal membuatlan ia lebih mahal berbanding makanan bukan halal, ini menyebabkan berlakunya penipuan pada label makanan halal.

Penggunaan daging anjing adalah dilarang dalam Islam dan ia kurang dibincangkan berkaitan penipuan mencampur bahan dalam makanan halal.. Walaubagaimanapun,menurut laporanyang telah dikeluarkan, daging anjing dijadikan makanan oleh warganegara Vietnam dan Myanmar yang bekerja di Malaysia.Penggunaan daging anjing juga berluluasa di negara China, vietnam dan Korea Selatan.Tambahan pula,anjing liar yang mudah didapati tanpa perlu membayarsebarang harga di serata negara membuatkan ia sangat berpotensi untuk menjadi salah satu punca penipuan dalam industri makanan halal.

Menurut kajian, lima ujian reaksi rantai polimerase (PCR) telah dicadangkan untuk mengesan daging anjing. Walau bagaimanapun, kebanyakan ujian yang telah direkodkan mensasarkan saiz amplicon yang lebih panjang (≥213 bp) dan tidak satupun dari mereka diuji dalam keadaan matriks makanan yang kompleks. Amplikon dengan saiz yang lebih panjang boleh mengalami kerosakan semasa memproses makanan dan menyebabkan

pengesanan "negatif salah " dalam penilaian akhir. Bagi mengatasi kekangan ujian yang sedia ada, kami telah membangunkan teknik "short amplicon-length conventional PCR ", “PCR-RFLP” dan” ” masa-sebenar PCR, mensasarkan bahagian 100-bp mitokondria cytochrome b gen. Bagi mengetahui sejauh mana spesifiknya ujian tersebut, ia diuji dengan menggunakan DNA yang diekstrak daripada anjing dan daging biasa temasuk hidupan darat , akuatik , burung dan tumbuhan. Kestabilan analisa diuji di bawah keadaan pemprosesan makanan yang berbeza, termasuk pendidihan," autoclave" dan gelombang mikro di dalam keadaan mentah, campuran dan makanan komersial dan ia didapati sangat stabil. "PCR" konvensional yang telah dikembangkan berjaya mengenalpasti 0.1% kepada 0.2% (w/w) daging anjing dari sampel dicampur dan komersial, membuktikan kestabilan dan kepekaan di bawah matriks kompleks. Produk PCR di buktikan dengan mencernakan produk menggunakan “*AluI* restriction enzyme" yang menhasilkan 51-, 30- dan 19-bp serpihan yang telah berjaya dipisahkan oleh "experion bioanalyer". Kepekaan dan sensitiviti PCR-RFLP adalah 0.0001-ng DNA daging anjing di bawah "pure state" dan 0.01% (w/w) daging anjing didapati dalam formulasi burger ayam dan daging. Sebagai penutup, penyelidikan TaqMan real-time PCR telah dibangunkan dan didapati sangat stabil dan sensitif dalam matriks asli dan kompleks. Manakala real timePCR yang telah di kembangkan berjaya mengesan 0.01% (w/w 0.0002 ng DNA) daging anjing dalam nuget ayam komersial. Sebanyak enam produk makanan yang dibeli dari kedai-kedai yang berbeza di Malaysia telah diuji dan tidak ada penipuan menggunakan daging anjing dapat di kesan.

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LIST OF ABBREVIATIONS

CE	Capillary Electrophoresis
CNT	Carbon Nanotube
Cob	Apocytochrome b
CTAB	Cetyl Trimethyl Ammonium Bromide
Cytb	Cytochrome b
DBKL	Dewan Bandaraya Kuala Lumpur
ELISA	Enzyme Linked Immuno Solvent Assay
EU	European Union
FAM	6-carboxyfluorescein
FRET	Fluorescence Resonance Energy Transfer
FSA	Food Standards Agency
GC-MS	Gas chromatography-mass spectrometry
GHP	Good Hygiene Practices
GMO	Genetically Modified Organism
GMP	Good Manufacturing Practices
HACCP	Hazard Analysis Critical Control Points
HCP	Halal Control Points
JAKIM	Malaysian Department of Islamic Development
MAG	Monoacylglycerol
MDTCC	Ministry of Domestic Trade, Cooperative and consumerism
MS	Mass Spectrometry
MWNT	Multi-Walled Nanotubes
NCBI	National Center for Biotechnology Information
OC	Osteocalcin
PAb	Polyclonal Antibody
PCR	Polymerase Chain Reaction
QDs	Quantum Dots
RAPD	random amplified polymorphic DNA

RFLP	Restriction Fragment Length Polymorphism
SPME	Solid-Phase Micro-Extraction
SS	Species specific
SWNT	Single-Walled Nanotube
TAG	Triacylglycerol
TAMRA	6-carboxytetramethyl-rodamine
TBE	Tris Borate buffer
TET	tetracholro-6-carboxyfluorescein
VOC	Volatile Organic Compound
2-DE	Two-Dimensional Electrophoresis
3D	Three- Dimensional

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CHAPTER 1
INTRODUCTION

1.1 Research Background

1.1.1 General background

The authentication of meat species is an increasing concern and is an integral part to ensure quality foods which must comply with health, religious faith, personal budget and of course fair prices (Ali, Hashim, Mustafa, & CheMan, 2012; Tanabe et al., 2007). The newly emerging "zoonotic threat" such as bovine spongiform encephalopathy (bovine species) (Brown, Will, Bradley, Asher, & Detwiler, 2001), human immunodeficiency virus (chimpanzee species) (Girish et al., 2004), H5N1 virus (avian species) (Beigel et al., 2005) and food allergens such as hazelnuts which may be a threat to certain consumers sensitive to this specific allergens (Piknová, Pangallo, & Kuchta, 2008) have tremendously intensified the need of identification and assignment of ingredient species in foods. According to European law, food manufacturers must declare and clearly label ingredients used in the preparation of both raw and finished foods (Commission, 2002). Most of the countries have legislations as well as regulatory and certification bodies to enforce the food labeling laws from farm to fork (Ali, Hashim, Dhahi, et al., 2012; Ali, Hashim, Mustafa, & Che Man, 2012; Ali, Kashif, et al., 2012; Murugaiah et al., 2009; Musa & Jalil, 2012; Tanabe et al., 2007).

"Halal" logo on food products is trusted by 1.8 billion Muslims of the globe and it defines that the products are prepared following the Shariah law of Islam for hygienic foods, and Muslims have no religious obstructions to consume them (Ali, Hashim, Dhahi, et al., 2012). However, the inherent nature of their specialized supply chain, preparation and storage requirements have made them costlier over their non-halal

counterparts (Ali, Hashim, Mustafa, & CheMan, 2012; Che Man, Aida, Raha, & Son, 2007; Murugaiah et al., 2009; Yusop, Mustafa, Che Man, Omar, & Mokhtar, 2012). Consequently, fraudulent labeling of halal brands is frequently taking place.

Canine or dog meat adulteration is a less discussed issue. However, it has been consumed for decades in many parts of the world, such as South Korea, China and Vietnam (Podberscek, 2009). Recently, reports have been made that foreign workers of Vietnam and Myanmar origins consume stray dogs in Malaysia (Nagpal, 2008). Since stray dogs are widely available in many parts of the world and dog meats do not have any market value (Kumarapeli & Awerbuch-Friedlander, 2009; Totton et al., 2010), we are prompted to take them as a potential adulterant in halal meats and meat products.

1.1.2 Molecular biomarkers and species identification schemes

Lipid (Rohman, Sismindari, Erwanto, & Che Man, 2011), proteins (Asensio, González, García, & Martin, 2008), and nucleic acids (DNA and RNA) (Ali, Hashim, Mustafa, & CheMan, 2012) are the key biomarkers for the molecular identification of food species. For species detection in foods, lipid-based biomarkers are based on the positional analysis of fatty acid triacylglycerol (TAG) and 2-monoacylglycerol (2-MAG) (SzabO, FEBel, SugAR, & RomvARi, 2007). But lipid based methods have drawbacks in species authentication due to the alteration of the positional distribution of fatty acids with TAGs and MAGs during the cooking process of foods (Ali, Kashif, et al., 2012; Marikkar, Ng, & Man, 2011). In certain literatures, it has been described for the utilization of protein biomarkers for animal species authentication using different techniques such as electrophoretic (Montowska & Pospiech, 2007), chromatographic (Chou et al., 2007) and spectroscopic (Ellis, Broadhurst, Clarke, & Goodacre, 2005). However, the potentialities of protein based approaches have been restricted due to the

denaturation of soluble protein during the thermal treatment of process foods. Furthermore, analyses of antibodies raised against a specific protein by immunoassays have the possibility of interruption by the cross-reactions of the closely related species, leading discrimination failure (Ayaz, Ayaz, & Erol, 2006). Recently, to overcome these limitations DNA based assays have drawn major importance in species authentication both from raw or processed foods. The DNA based biomarkers have enough discriminating power to distinguish varieties of animal or plant species depending on the variation of the sequence in the genome. DNA is more resilient to destruction by food processing (particularly cooking and sterilization) than other marker substances. Thus, DNA based molecular identification schemes have been proven to be the methods of choice because of their specialized features of the molecule itself, such as codon degeneracy, higher stability and universality in all tissues and cells (Ali, Hashim, Mustafa, & CheMan, 2012). Therefore, several DNA based biomarker assays such as species specific PCR (Martín et al., 2009), PCR-RFLP (Ali, Hashim, Mustafa, & Che Man, 2012), SYBR green real-time PCR (Farrokhi & Jafari Joozani, 2011), molecular beacon real-time PCR (Yusop et al., 2012), TaqMan probe real-time PCR (Ali, Hashim, Dhahi, et al., 2012) and nanoparticles sensor (Ali, Hashim, Mustafa, Man, & Islam, 2012; Ali, Mustafa, Hashim, Man, & Foo, 2012) have been proposed for the species authentication in foods. For better utilization of these DNA based assays, short-length DNA markers with target species fingerprints are especially interesting, since they allow the target species detection under extremely processed or compromised states. Literature surveys revealed that five different DNA-based methods have been documented for dog-species detection (Abdel-Rahman, El-Saadani, Ashry, & Haggag, 2009; Abdulmawjood, Schönenbrücher, & Bülte, 2003; Gao, Xu, Liang, Zhang, & Zhu, 2004; İlhak & Arslan, 2007; Martín et al., 2007). However, nearly all of them are based on longer DNA target amplification which may breakdown under compromised states

(Abdel-Rahman, El-Saadani, Ashry, & Haggag, 2009; Abdulmawjood, Schönenbrücher, & Bülte, 2003; Gao, Xu, Liang, Zhang, & Zhu, 2004; İlhak & Arslan, 2007).

1.2 Problem Statements

Morphological, lipid or protein based species-identification schemes are not trustworthy and reliable since these biomarkers easily undergo modification during cooking and food processing steps (Ali, Kashif, et al., 2012; Ayaz et al., 2006). DNA based methods are trust worthy but the reliability depends on the target DNA-lengths, e.g. longer DNA targets breakdown more easily than the shorter ones (Ali, Hashim, Mustafa, & Che Man, 2012). Nearly all of the documented PCR assays for dog-species authentication are based on longer-size DNA targets and they have not been tested under complex food matrices and processed states (Abdel-Rahman, El-Saadani, Ashry, & Haggag, 2009; Abdulmawjood, Schönenbrücher, & Bülte, 2003; Gao, Xu, Liang, Zhang, & Zhu, 2004; İlhak & Arslan, 2007). Consideration of additives and matrices are important factors in PCR-based detection since PCR reactions are inhibited by certain factors, resulting in false negative detection (Bottero & Dalmasso, 2011; Di Pinto, Forte, Conversano, & Tantillo, 2005). Additionally, reduction of amplicon-length often compromise polymorphism and specificity (Ali, Hashim, Mustafa, & CheMan, 2012). Thus, there is a huge opportunities and challenges in the development of short-length PCR biomarkers and its validation under complicated food matrices.

1.3 Objectives of the Research

1.3.1 General objective

The objective of the present research is to develop and characterize short-length DNA-based molecular techniques for the detection and quantification of canine species under raw, processed and commercial matrices of processed foods.

1.3.2 Specific objectives

a. To develop short-length DNA biomarkers with adequate fingerprints for canine species.
b. To test the potentiality of biomarkers for sensitive detection of canine-species under various food processing treatments and complex matrices using PCR-based techniques.
c. To analyze assay performance and validity for the screening of processed meat products for the detection and quantification of canine-adulteration.

1.4 Scope of Studies

1.4.1 Development of short-length DNA biomarkers

DNA based assay platforms are promising field of study not only for food species detection but also for molecular detection of pathogens in agriculture, food analysis, biodiagnostics, environmental monitoring, bioterrorism, and forensic analysis (Iwobi, Huber, Hauner, Miller, & Busch, 2011; Jung, Mun, Li, & Park, 2009; Teletchea, Maudet, & Hänni, 2005). However several studies demonstrated that DNA based assays with longer targets are prone to break down under extensive food processing state causing failure of the assay (Ali, Hashim, Mustafa, & Che Man, 2012; Rojas et al.,

2010). In contrast, short length DNA biomarkers amplify more efficiently, separate with higher resolution and shows better recovery in degraded sample analysis (Ali, Hashim, Mustafa, & CheMan, 2012). Furthermore, DNA biomarkers of short lengths have huge interests for biosensor (Jung et al., 2009), biochip and forensic applications because of their excellent stabilities in hostile environments (Aboud, Gassmann, & McCord, 2010). Moreover, multicopy mitochondrial gene facilitates the forensic or highly degraded samples analysis due to the extra ordinary stability under harsh condition (Butler, 2006). Mitochondrial multicopy gene target provide available DNA template for hybridization and increases the assay sensitivity (Tanabe et al., 2007). However, shorter amplicon can be hardly achieved without compromising specificity (Hird et al., 2006). Hence, for achieving the objective of a shorter DNA target for canine species detection, primers will be designed using mitochondrial multicopy cytochrome b gene. Primary screening for the specificity of the assay will be done by using BLAST analysis tools of publicly available data base of National Center for Biotechnology Information (NCBI). Furthermore, for higher specificity DNA sequences of potential animals, plants and fish species will be retrieved and aligned to get the hyper variable region. The alignment result will also allow us to calculate the oligonucleotide mismatch in the primer binding site to prevent the nonspecific amplification of the assays. Thus, the proposed work will develop short amplicon based mitochondrial DNA biomarkers assay for the detection of the canine species either from raw or processed foods.

1.4.2 Assessment of biomarker-specificity using PCR-based techniques

The evaluation of biomarker specificity should be analyzed through a well-known method to avoid ambiguity. PCR is a well-known molecular tool for the identification of meat-species in foods (Ali, Hashim, Mustafa, & Che Man, 2012). It is based on the in vitro amplification of the specific DNA target using hybridization of the

oligonucleotide primers on the flanking region. The highly specific primers distinguish the target where the specific fragment application can be determined by certain electrophoresis technique. The separation of the DNA fragment using agarose gel is the simplest PCR strategy for determination of the species of interest. The digestion of PCR products with restriction endonuclease and PCR-RFLP assay provide more authentic species detection platform which can be further enhanced by application of capillary electrophoresis (Mafra, Ferreira, & Oliveira, 2008). However, the detection of specific target by traditional gel image provide only a simple yes or no answer to the question 'Is this foodstuff or ingredient what I believe it to be?' (Woolfe & Primrose, 2004); but sometime, it is necessary to know the amount of adulterant present in the food. The real-time PCR assay allows the quantitative analysis of the specific target using a fluorescent reporter molecule (Wiseman, 2002). Therefore, the proposed research will analyze the performance of the designed DNA biomarkers with PCR, PCR-RFLP, and TaqMan real-time PCR assay both for qualitative and quantitative analysis of canine DNA in foods.

1.4.3 Assay validation and food analysis

The major drawbacks of the lipid and protein based assays is the specificity and stability of the assay under food processing steps. Hence, primarily the performance of the PCR assay will be tested in real run using DNAs extracted from raw canine meat as well other commonly used meat animals, fish and plant species. Subsequently the assay will be tested under different food processing steps such as boiling, autoclaving; oven heating etc to realize the stability of the canine specific DNA target. The assay specificity and sensitivity will be further extended by testing under binary or ternary admixed analysis using dog meat with other commonly used meat species. For protection of the consumer rights from fraudulent labeling of the foods, it is very

important to prove the meat species of high commercial value declared are not substitute, partial or entirely, by other lower value species in the process foods (Mafra et al., 2008). The lower valued meat such as from canine species may be harmful for health and act as source for certain diseases. It may also be a potential source for food borne allergen. The substituted lower cost meat may affect consumer lifestyles, such as vegetarianism, or religious practices. For example, the Jews and Muslims have the food taboo for pork meat consumption. Furthermore, the recent meat scandal on different food products made it essential to test what are we eating? (Ali, Razzak, & Hamid, 2014). However, the presence of various additives and inhibitors in commercial meat and food products might prevent the primer binding at specific sites and reduce the amplification efficiency, diminishing the sensitivity and specificity of the assay (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Calvo, Zaragoza, & Osta, 2001; Di Pinto et al., 2005). Therefore, the developed assays will be validated by preparing model experimental samples of certain highly valued commercial food products such as frankfurter, meatball, burger, nuggets, etc. Thus, for the protection of consumer's health and rights the assays can be used as laboratory tools in the food industries as well as in Halal food authentication laboratories.

1.5 Thesis Organization

This thesis has been organized into eight chapters numbered as chapters 1-8. The first chapter describes the research background, problem statements, objectives and the scope of the present work. It focused the importance of the present study, with a brief description of the limitation of the previous methods. We have outlined here the approaches we have adopted to overcome the limitations of the documented methods to bring some innovation in the new method that we have developed here. The second chapter presents the review of general understanding and literature outcomes. It highlighted the different forms of food adulterations, molecular biomarkers and detection methods for authentication. The current status of canine meat uses around the globe has also been outlined.

In the third chapter, we described the development of canine biomarkers for conventional PCR-based detection, and validated its application for the analysis of commercial frankfurter. We have succeeded to publish the chapter findings in the Food Analytical Methods (Food Analytical Methods 7(1), 234-241).

In the fourth chapter, we have extended the application of the method that we developed in the third chapter for the screening of meatballs, a popular meat products consumed around the globe. We have formulated both model and commercial meatballs and showed the assay validity for canine authentication in meatballs. This chapter has been published in the Meat Science (Meat science, 97(4), 404-409).

The fifth chapter is the further extension for the application of method developed in the third chapter for the analysis of canine meat contamination in the popular Arab and Indian cuisine. The chapter output have been presented in two international conferences, namely 1st International Conference on Molecular Diagnostics and Biomarker Discovery (MDBD 2013) and International Conference on Food Innovation 2014

(INNOVAFOOD 2014). Additionally, a full-length paper has been submitted to International food Research Journal.

The sixth chapter has described the development of a PCR-RFLP assay which is the shortest-length in its kind for the authentication of canine species. The assay was based on lab-on-chip microfluidic technique incorporated with bioanalyzer for the separation of short-length nucleic acid fragments. The assay validity has been tested for the screening of commercial burgers and results have been published recently in the Food Analytical Methods (DOI: 10.1007/s12161-015-0090-1).

The seventh chapter explained the development of a quantitative TaqMan probe real-time PCR assay for the detection and quantification of canine species under complex matrices such as processed foods. The assay validity has been tested for canine species determination in commercial nugget.

Finally, the eighth chapter discusses the finding summary of the overall research and has outlined the future research in this particular field.

CHAPTER 2
LITERATURE REVIEW

2.1 Food Adulteration

Food adulteration is defined as the deliberate act of degrading the quality of food products by fraudulent admixing or substitution of low-grade ingredients for its higher value counterparts to harness financial gain or additional profit.

According to the Federal Food, Drug, and Cosmetic (FD&C) Act (1938) of the United States (Frank & Hahn, 2003), "adulterated" foods fall under any of the following criteria:

a) Food that bears or contains any "poisonous or deleterious substance" which may lead to injury or harm to health.

b) Food that contains a pesticide; chemical residue; food additives or new animal drug those are unsafe for human health.

c) Food that consists, in whole or in part of any filthy, putrid, or decomposed substance; or it has been prepared, packed, or held under unsanitary conditions such as infested with insect, rodent, or bird. Thus it becomes contaminated with filth which makes it injurious to health.

d) Food that is, in whole or in part, the product of a diseased animal or of an animal which has been died due to causes other than slaughtering.

e) Container of the food composed of in whole or in part of any poisonous or deleterious substance which may render the food contents injurious to health.

f) The irradiation process of the food was not performed under the inconformity with the regulation of radiation or exemption of law.

g) Food with recommended labeling of dietary ingredients which may lead to significant illness or injury towards health. For example, aristolochic acids containing foods or dietary supplements may link to kidney failure and it has been banned.

h) Food where any valuable constituent has been omitted or abstracted in whole or in part or replaced with another substance; or damage or inferiority has been concealed in any manner. The food with a substance that has been added to increase the product's bulk or weight, reduce its quality or strength, or make it appear of greater value.

The U.S. Department of Agriculture defines Meat "Adulteration" under the "Definitions" section of the Meat Inspection Act 21 USC Sec. 601(FDA, 2009). According to this meat will be considered as ''adulterated'' if any carcass, part thereof, meat or meat product fall under one or more of the following circumstances:

a) If the meat or meat product bears or contains any poisonous or deleterious substance which may render it injurious to health.

b) If the food bears or contains any added poisonous or deleterious substance such as pesticide chemical; raw agricultural commodity; food additive; or a colour additive (under certain rule) which will lead the food unfit for human consumption.

c) If the meat consists in whole or in part of any filthy, putrid, or decomposed substance or is for any other reason unsound, unhealthful, unwholesome, or otherwise not suitable for human consumption.

d) If the meat has become contaminated with filth and whereby it may have been rendered injurious to health due to the insanitary conditions of preparation and packaging.

e) If the meat or meat product is made of animal dies of other causes but not slaughtered; or if it has been intentionally subjected to radiation, unless the use of the radiation was in conformity with a regulation.

However, the term "food adulteration" or "meat adulteration" has not been clearly defined by the European Union (EU) (Avery, 2014). The EU food law preserves consumer rights by preventing fraudulent or deceptive practices; the adulteration of

food; and any other practices which may mislead the consumer" (Avery, 2014). The food industry should secure the quality of the foods with traceability. The foods should be hygienic with proper labeling of the ingredients (Avery, 2014). Thus the labeling of food, presentation, advertising or packaging "shall not mislead consumers" (Johnson, 2014). However, The United Kingdom's Food Standards Agency (FSA) illustrated the "food fraud" as the deliberate placement on the market, for monetary gain, with the objectives of deceiving the consumer by two major ways such as i) selling of the food products which are unfit and harmful for human health and ii) deliberately mislead the consumer by misdescription of food, such as substitution of the products with a cheaper alternative (Johnson, 2014).

Finally, according to the Department of Standards Malaysia (MS, 2009) in Islamic Shariah law Muslims are permitted to eat and drinks if the foods or drinks and/or their ingredients fulfill criteria as given below:

a) It does not include any parts or products of animals that are non-halal by Shariah law or any parts or products of animals which are not slaughtered according to Shariah law;

b) The food should be non-poisonous, non-hazardous for health and safe for human consumption;

c) It does not contain najis (dogs and pigs descendents) according to Shariah law;

d) The food products should not be prepared, processed or manufactured using equipment contaminated with najs according to Shariah law;

e) Food products should not contain any human parts or its derivatives that are not permitted by Shariah law such as urine, blood, vomit, pus etc.

f) During food products preparation, handling, processing, packaging, storage and even in distribution, the products should be physically separated from any other foods that does not meet the requirements stated in items a), b), c), d) or e) or any other things that have been decreed as najis.

2.2 Misbranded Food

According to the United States Codes "21 U.S.C. §343" (NDSU, 2010), food is considered as mislabeled or misbranded if it falls under one or more of the following criteria:

a) If food container is made, formed, or filled with deceptive or it's labeling is false or misleading.

b) If the food package label does not include the manufacturer information with name and place including the packer or distributor with accurate quantity (weight, measure, or numerical count).

c) If the nutritional composition of the food offered for human consumption or sale is not properly stated. For example the total number of calories derived from any source, and derived from the total fat, in each serving size; the amount of total fat, saturated fat, cholesterol, sodium, total carbohydrates, complex carbohydrates, sugars, dietary fiber, and total protein contained in each serving size; and any vitamin, mineral; the number of servings per container.

d) Placement of word, statement, or other information required by law misleads the consumer while purchasing the product. For example, labeling is not prominently placed thereon with such conspicuousness (as compared with other words, statements, designs, or devices, in the labeling) which cannot be read and understood by the ordinary customer while purchasing or in use.

e) Trading of food under the name of another food or if it is an imitation of another food, without labeling the word "imitation."

f) The labeling of the food does not contain common or usual name of the food. If fabricated from two or more ingredients, the common or usual name of each such ingredient should be included. Furthermore, if the food purports to be a beverage containing vegetable or fruit juice, a statement with appropriate prominence of the total percentage of such fruit or vegetable juice contained in the food.

According to EU food law, there is no definition of the “misbranded food” (Brivio & Apostola, 2014), however the new EU Regulation for the protection of consumer right and health included the following labeling condition of the foods:

a) The processed food must contain nutritional information;

b) The unprocessed meat either from sheep, pigs, goats or poultry must have labeling of origin;

c) Clear and integrated information of allergens of the food ingredients such as nuts, soy, gluten, and lactose of the repacked products should be highlighted with emphasized font, style or background color:

d) Enhanced legibility with minimum font size of the text;

e) Non pre-packed foods those sold in restaurants and cafés should also provide information on allergens;

f) Product sold online or distance and in the shop selling should contain the same labeling;

g) Tailored nanoparticles based ingredients should be enlisted in the labeling of the products;

h) Exact information of the vegetable originated refined fats and oils;

i) For 'Imitation' foods, indication of substitute ingredient;

j) Clear indication of "formed meat" or "formed fish"; and defrosted products.

2.3 Effect of Food Adulteration or Misbranding

Fraudulent adulteration of food item with serious human health hazard is not a recent problem. For example, certain food additives used in the 18th or 19th centuries such as poisonous alum and chalk was added to the flour to make bread (Tähkäpää, Maijala, Korkeala, & Nevas, 2015). Intentional melamine-tainted infant formula of a Chinese company had a direct link to the public health effect causing death of six children and about 300 000 children became sick (Guan et al., 2009; Jia & Jukes, 2013). In 1981, admixture of vehicle oil with the oil for human consumption in Spain caused toxic oil syndrome, which causes the suffering of ≥20 000 consumer leading to death of 1663 (Borda et al., 1998). More recently sunflower adulteration with mineral oil has been detected in certain European countries (Tähkäpää et al., 2015).

Recent decade fraudulent description of meat and meat products was top food industrial news. For instance, Japanese meat companies mislabelled imported beef as domestic meat after subsequent outbreak of Bovine Spongiform Encephalopathy infection in 2002 (Yeboah & Maynard, 2004). In China, non-approved colouring material (Sudan) was used in meat products in 2005 (Jia & Jukes, 2013). In the UK, diseased bleached poultry was diverted back into the retail food chain in 2007 (Reynolds, 2008; Tähkäpää et al., 2015). In Northern Ireland, poultry and beef of unknown source were illegally repackaged and consigned for human consumption on the market (Reynolds, 2008). In France 2006, corned beef was infused with unfit meat and false declaration was made as Halal Food. In the same year, in Germany rotten meat scandal occurred where around 150 tonnes of rotten meat was distributed to restaurants nationwide (Bosley, 2007). In numerous EU member states, fraudulent labelling beef products draw tremendous attention world wide due to the detection of ≥ 60% of horse meat in beef products in 2013 (FSA, 2013).

The victims of fraudulent adulteration by different food frauds are not only the consumers but also governments, and even some time companies (Figure 2.1) (Kulas, 2014). The consumers while purchasing the product, believed in the ingredients content stated in the product. The companies and suppliers engaged with the manufacturing using the fraudulent products believed that they were using wholesome raw ingredients. The government consider that the existing food quality controls are monitoring the bulk adulterated products. Thus French Finance Ministry calculated that horse meat scandal affected over 4.5 million processed beef products, equalling about 1,000 tons of food and affected all form of three food adulteration victim consumers, governments, and companies by the food frauds (Ruitenberg, 2013).

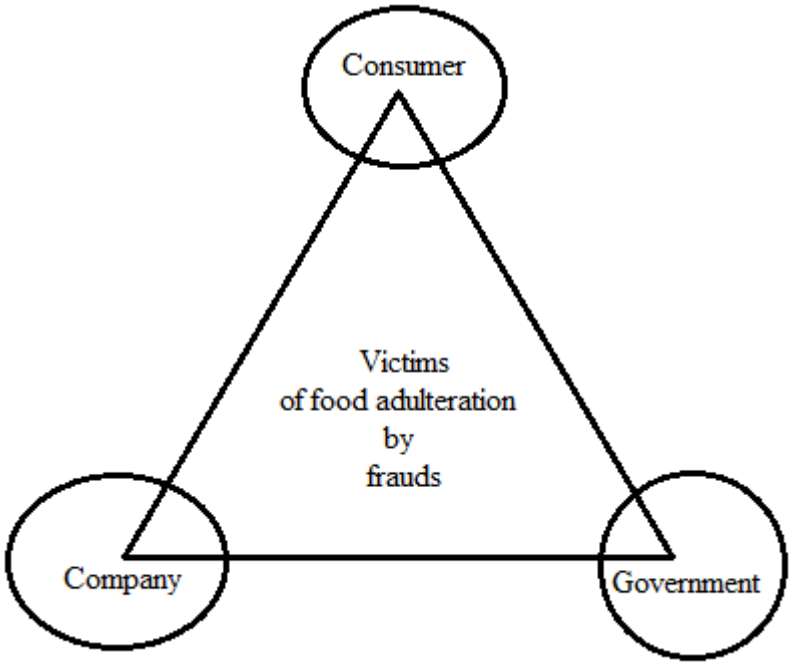

Figure 2.1 Victims of food adulterations.

2.4 Halal Foods and Authentication Chain

2.4.1 Halal foods

In Islam, the dietary rules are defined by Islamic Shari'ah law following the Quran (the divine book), Hadith (the compilation of the traditions of Prophet Muhammad) and Fiqah (consensus opinions of a group of Islamic scholars). "Halal" is an Arabic word used in scripture to define lawful or permitted things for the Muslim. Thus according to the Department of Standards Malaysia (MS, 2009) in Islamic Sharia law the source of the animal origins halal foods may derived from two categories such as i) Land animals and ii) aquatic animals which are described below

i) Land animals

All land animals are halal as food except the following:

a) Animals that are not slaughtered according to Shariah law;

b) Najis/mughallazah animal, i.e. pigs and dogs their descendants;

c) Animals with long pointed teeth or tusks which are used to kill prey such as tigers, bears, elephants, cats, monkeys, etc.;

d) Predator birds such as eagles, owls and etc.;

e) Pests and/or poisonous animals such as rats, cockroaches, centipedes, scorpions, snakes, wasps and other similar animals;

f) Animals that are forbidden to be killed in Islam such as bees, woodpeckers (hud-hud), etc.;

g) Creatures that are considered repulsive such as lice, flies, etc.;

h) Farmed halal animals which are intentionally and continually fed with najs; and

i) Other animals forbidden to be eaten in accordance to Shariah law such as donkeys and mules.

ii) Aquatic animals

Aquatic animals are those which live in water and cannot survive outside it, such as fish. According to the Islamic prospective, all aquatic animals are halal except those that are poisonous, intoxicating or hazardous to human health. In addition, animals that live both on land and water such as crocodiles, turtles and frogs are not halal. Furthermore, aquatic animals which are fed on najs or intentionally and/or continually fed with najs are not halal.

Processed foods will be defined as Halal (MS, 2009), if they meet the following criteria:

a) Food or its ingredients not be processed by using any components or products of animals that are non-halal by Shariah law or of halal food any components or products of animals that are not slaughtered according to Shariah law;

b) Food not be processed by using anything or in any quantity that is decreed as najs by Shariah law;

c) Processed food or its ingredients safe for human consumption, non-poisonous, or non-hazardous to health;

d) Food prepared, processed or manufactured using equipment and facilities that are free from contamination with najs; and

e) during its preparation, processing, handling, packaging, storage distribution and serving, it shall be physically separated from any other food that does not meet the requirements specified in items a), b), c) and/or d) or any other things that are decreed as najs by Shariah law.

2.4.2 Halal authentication chain

To safeguard consumer health, Hazard Analysis Critical Control Points (HACCP) (Codex Alimentarius Commission, 2003) is a globally recognized and applied quality assurance scheme within companies placed at different levels of agro-food supplying process. Thus for ensuring the halal status of the meats, a HACCP approach with several halal critical control points from the slaughterhouse to the consumers was proposed (Bonne & Verbeke, 2008). For a total or integrated halal quality control approaches in accordance with HACCP principles, Zadernowski, Verbeke, Verhé, and Babuchowski (2002) and Snijders and Van Knapen (2002) bring forward the focus on upstream pre-harvest production and downstream retailing from the farm to fork chain. However, most of the recent meat scandals are associated with the minced or process food products. Hence, the time demanding halal critical control points (HCPs) for authentication process or supply chain with an integrated halal transportation system and quality assurance systems with proper laboratory protocol have been proposed (Figure 2.2). Thus it will improve meat or meat products authentication approach with comprehensive tools for better assurance and safeguarding standards, related to animal welfare and certified production processes of halal foods.

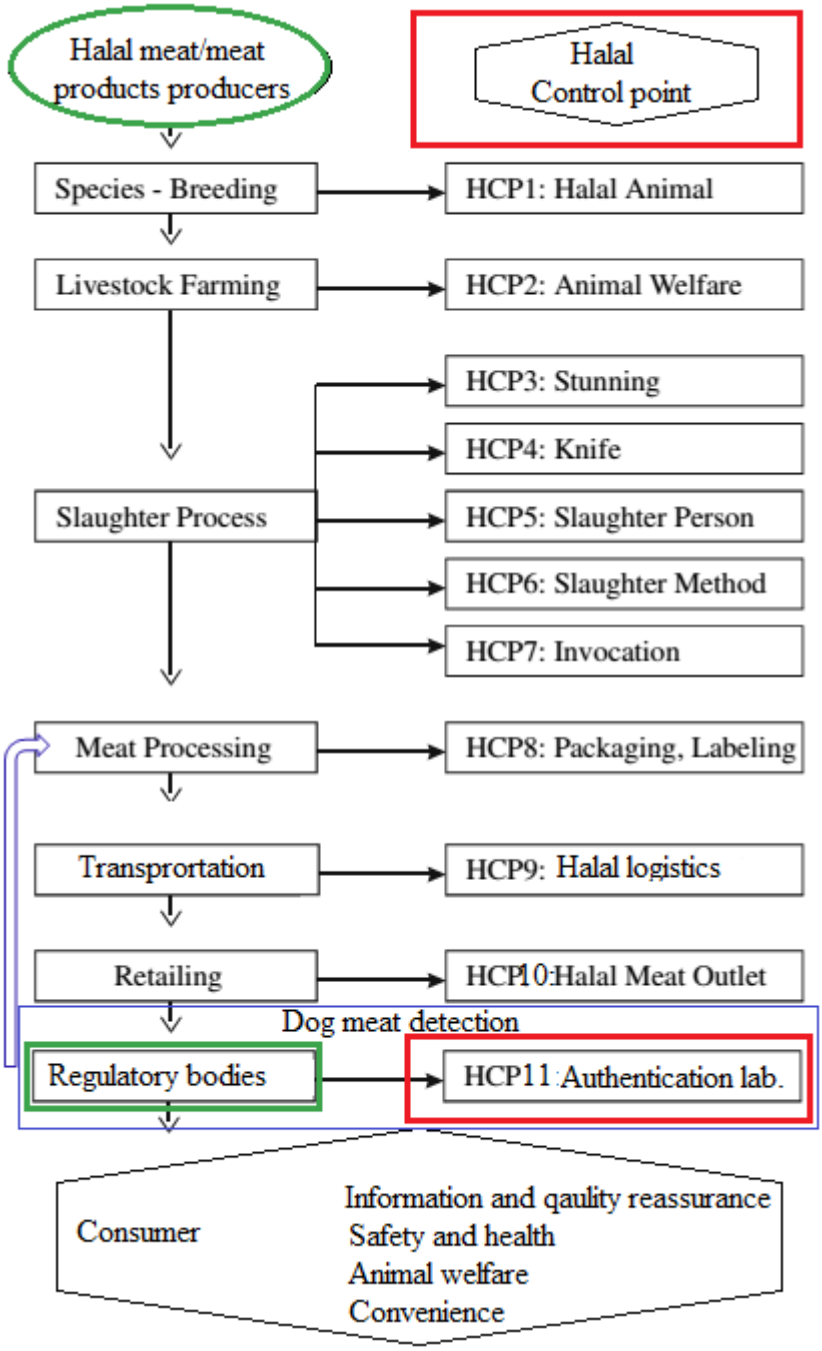

Figure 2.2 Halal meat/meat products authentication chain along with different Halal Control Points (HCP), adapted from (Bonne & Verbeke, 2008).

2.5 Malaysian Halal Food Standard

To verify the halal foods and create halal food awareness among the producers, distributors, importers, restaurants and hotels Malaysian Department of Islamic Development (JAKIM) has been playing a great role since 1982. Thus from the Malaysian governments, a precise and clear outline for the halal foods was presented for first the time in August 2004 as Malaysian Standard: Halal Food-Production, Preparation and Storage-General Guidelines (MS 1500:2004) (Samori, Ishak, & Himmah, 2014). The standard was developed according to the ISO methodologies and was widely recognized and accepted globally by different halal certification bodies. It was the first prescribed halal standard with practical guidelines for the food manufactures including the preparation and handling of halal foods (Samori et al., 2014). It described the selection of raw materials for halal foods along with the distribution and marketing activities. The MS 1500:2004 was revised in 2009 by adding compliance with Good Manufacturing Practices (GMP) and Good Hygiene Practices (GHP). However, for the production of halal foods in Malaysia the basic requirements based on MS 1500: 2004/2009 are mentioned below:

a) Sources of halal food and drinks should be halal animal and plant based,

b) Slaughtering of halal animal should be done separately from the non-halal animals,

c) Product processing, handling and distribution should comply with halal regulations,

d) Product storage, display and serving for instance equipments, machineries and other materials used must not be made of non-halal materials,

e) Hygiene, sanitation and food safety should comply with the concept of halalan tayyiban,

f) Packing and labeling must be carefully evaluated by JAKIM,

g) Legal requirements which in line with MS 1500: 2004 revised 2009.

Physical separation of Halal food products from non-halal is important from production, preparation, handling and storage (Janis, 2004), with subsequent labeling. Recently Malaysian government has introduces halal act through the Parliament with Trade Descriptions 2011, Food Act 1983, Animal Rules 1962, Consumers Act and Customs Act 1998 with a description of import and export of the halal meat (Samori et al., 2014). Furthermore for purchasing the foods without doubt, halal logo is incorporated with the verification process for Malaysian halal certification. JAKIM act as a coordinating body for halal certification in Malaysia with a standard halal logo, which resolve the uncertainty and misconception of halal products from any Malaysian outlets. Halal certificate is a dual concerned with product contents as well as ensure the products hygiene and safety. Thus the halal logo acts absolutely will add value to play a great rule in the halal industry (Shafie & Othman, 2006). Recently, the Department of Standards Malaysia introduced the MS 2400:2010: ”Halalan-Toyyiban Assurance Pipeline standard”, introducing the certification of halal logistics operations in Malaysia, which included the transportation process along with warehouse maintenance and retailing (Tieman & Ghazali, 2014).

2.6 Future of Halal Food Market

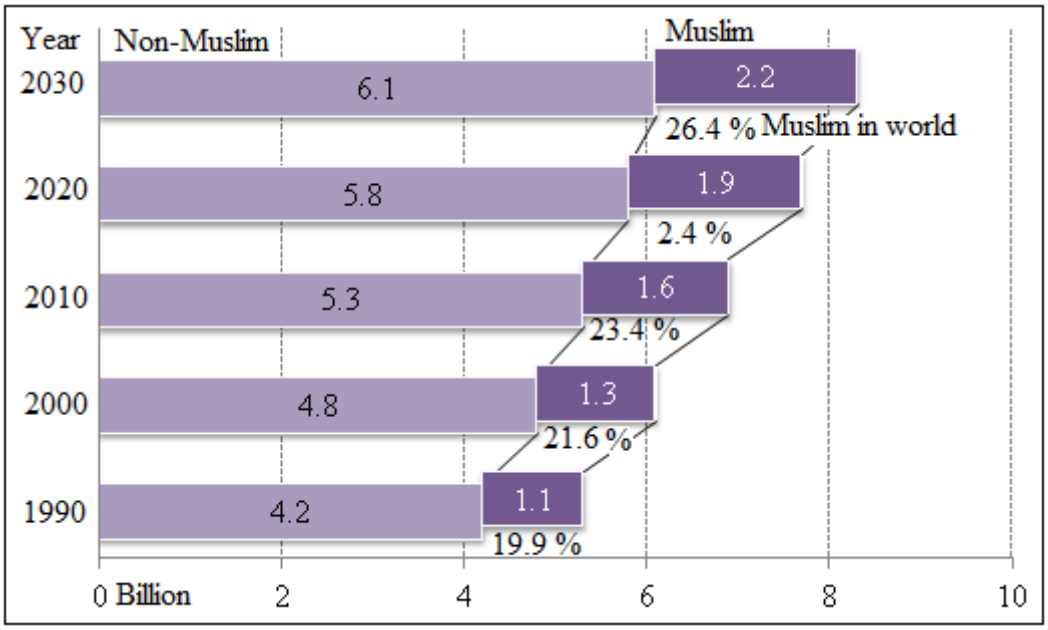

Figure 2.3 Projection of future world Muslim population by the year 2030; Adapted from (Pew Research, 2011).

The Muslim population in the world is growing twice faster rate than the non-Muslim population with an annual growth rate of 1.5% and 0.7% respectively. The world Muslim population is projected to be increase about 35% in next two decades. According to the current growth trend, the Muslim population will reach 2.2 billion in the next 20 years, constructing 26.4% of the world human population (Figure 2.3) (Pew Research, 2011). This huge Muslim population is the potential customer for the Halal branded products. The estimated annual growth of halal food consumption is about 16% and 63.25% of the total market value of the Halal food products was Asia in 2010 (Figure 2.4). By the year 2030, more than 50% (1.3 billion) of the projected Muslim population in the world will be the resident of South Asia and Asia Pacific. Thus 40% of the future potential halal customers of the world will be from Indonesia, Pakistan, India, and Bangladesh from this South Asia and Pacific region. Furthermore, Muslim population will be doubled in America (From 5.25 million to 10.5 million) and nearly a

third increase (from 44.1 million to 58.2 million) in Europe, by the year 2030. Beside the Muslim population Halal food is one of the choices for no-Muslim consumer too due to ethical and safety issue. For instance, there are 2 million Muslim in UK, but the consumer of Halal meat is about 6 million. In the Netherlands, the estimated market value of Halal food products is about USD 3 billion on an annual basic where it includes the non-Muslim Dutch consumers (Hughes & Malik, 2014). Although only 20% of the global Muslim population from Arab and Middle East, however 50 % of the countries out of 10 with higher purchasing power belongs to these areas (Hughes & Malik, 2014).

Region	USD (billion)
Asia	418.1
Middle East/Africa	155.9
Europe	69.3
Americas	16.7
Australia	1.6
Total market	661.6

Figure 2.4 The market value of halal foods in different parts of the world in 2010, Adapted from Hughes & Malik (2014).

2.7 Significance of Halal Logo

"Halal" logo in the food products plays a significant role in food purchasing decision (Samori et al., 2014). For example, the Malaysian Muslims are highly confident with the halal logo from JAKIM's (Shafie & Othman, 2006). They will not buy the food products unless it is imprinted with the halal logo. Further, it has been reported that Muslim consumer is even not willing to buy foods from the non-Muslim premises even it displayed the halal logo, which may be a part of precaution of the consumers caution to various alarming cases of contaminated halal food (Samori et al., 2014). However, these reflected the consumer attitude towards the halal food emphasizing the value of the halal logo. JAKIM has taken step towards the caution of Muslim consumers by publishing a halal food directory and creating e-halal website. Furthermore, for solving e the Muslim consumer doubt it is establishing an SMS (Short Message Services) network.

2.8 Fraudulent Halal Business

The illegal business of non-Halal meats threaten the public health, ignores people religious beliefs and strike on the consumers trust. Fraudulent halal business have been globally spread and not confined to the Muslim populated country only. For example, the trade non-Halal meats was the third largest illegal trade in the UK and was estimated to be worth of USD 1.5 billion a year (Pointing & Teinaz, 2004). The fraudulent meat trades with no moral qualms had even spread certain disease such as New Variant CJD (Pointing & Teinaz, 2004). There are different types of fraudulent usage of Halal for business acquirement, for instance the use overdue or expired Halal compliance certificates by the companies or the use of forged certificates (Halim & Salleh, 2012).

In Malaysia, for more profit and to attract the Muslim consumer some of the restaurant owner used fake Halal logo. This fake Halal logo is used for displaying purpose in payment desk which is not provided by the JAKIM. This imitated logo is resemblance to the Halal logo issued by JAKIM and cannot be verified by the individual customer. Thus the Ministry of Domestic Trade, Cooperative and consumerism (MDTCC) have revealed six fake Halal logos in the market which were resemblance to the Halal logo provided by the JAKIM (Mustafa'Afifi et al., 2013). The worst issues for the consumers were presence of pig DNA or traces of alcohol which is strictly prohibited in Islam within the halal food products while repeated occurrence of this have been blown out Malaysian media (Mustafa'Afifi et al., 2013). A joint conduction of Buffet analysis in 2010 by MDTCC and JAKIM, revealed a tragic news for the Halal status of different Malaysian outlets, where 52% of (50 out of 96) the hotels and restaurants premises not fulfill Halal standard as denoted by JAKIM or State Islamic Religious Departments (JAIN). The observation includes the use of non valid Halal logo issued by JAKIM or JAIN, the use of doubtful raw materials, alcohol, and chicken from uncertain sources. It also includes the non halal certification of raw materials used, transportation and storage of halal and non halal raw materials together (Mustafa'Afifi et al., 2013). MDTCC have reported a total of 29 cases in 2012, where 1 case was settled with a compound of 3000 Malaysian Ringgit (RM). The rest were seized and their goods which were confiscated were values about 15000 RM (Halim & Ahmad, 2014).

2.9 Food Authentication Technology

For analysis of different food components including the halal authentication of the meat or meat products with different spectrum or images generated by certain analytical tools can be used (Manning & Soon, 2014). These spectral or image data known as fingerprinting can be three types namely a) spectral fingerprinting, b) chromatographic fingerprinting, and c) electrophoresis fingerprinting (Zhang, Zhang, Dediu, & Victor, 2011). These fingerprinting technologies are widely being used for the detection of food materials and components including the origin of the species such as animal, poultry and fish (Ali, Hashim, Mustafa, & Che Man, 2012; A. Arslan, Ilhak, & Calicioglu, 2006; Hubert et al., 2008; Zhang et al., 2011). Thus, the application of different technologies for food component analysis is included in Table 2.1 (Manning & Soon, 2014).

Table 2.1 Application of different technologies for the detection of food components, adapted from Manning & Soon (2014).

Component	Food products	Indicators	Detection technology
Origin	Mutton, tea, beer, wine, olive oil	Isotope indicators aromatic compound, microelements, water, protein, carbohydrate, lipid,	PCR, NMR, IR
Species/ Material	Animal, Poultry, Rice, Vegetable, Bird's nest, aquatic product	DNA, Lipid, Protein	Isoenzyme electrophoresis, PCR, RFLP, AFLP, RAPD, small sequence length polymorphism, SDS–PAGE,
Components	Beef, ham, fruit, milk, edible oil, tea, health products	Lipid, protein, lecithin, sugars, vitamins, organic acid,	MS, SDS–PAGE, NMR, IR, UV
Additives	Meat, milk, juice, processed food, , ice-cream, carbonated beverages	Antiseptic, nitrite, sufan, melamine, colorants, clenbuterol hydrochloride,	GC, MS , UV, LC
Objectionable constituent in processing	Barbeque, fried starch products, margarine	Fatty acids Benzopyrene	LC, MS, UV,

2.10 History of Canine Meat Consumption

According to the history of animal domestication, dog was first domesticated around 15,000 years ago in East Asia when major farmed animal like cattle, horse, sheep, goat, chicken (Savolainen, Zhang, Luo, Lundeberg, & Leitner, 2002) were under wild states. From ancient time to still today, regardless of aim of domestication, dog meat has been consumed by human being (Podberscek, 2009). The countries where dog meat has been used in food recipe are in Southeast Asia and Indochina, North and Central America, parts of Africa, and the islands of the Pacific (McHugh, 2004). In Europe, there were prevalent uses of dog meat during the Neolithic and Bronze Ages (Vigne, Guilaine, Debue, Haye, & Gérard, 2004). Nowadays, although dog meat was out lawed in certain countries and huge protection against dog eating by animal welfare group, still consumption of dogs are reported in Cambodia, China, Thailand, Vietnam and South Korea (Podberscek, 2009). In South Korea, dog was classified as domestic animals (Oh & Jackson, 2011) and was available in traditional meat market for sale (Figure 2.5 a) (Lee, 2008). Dog is commonly known as man's best friend in the West but in Cambodia Phnom Penh's is a popular dish made of canine meat (Figure 2.5 b) (Mee, 2009). There is no particular food rule in Cambodia and many Cambodians believe that dog meat is tastier than beef, chicken and pork. It is unknown when the Vietnamese started to eat dog meat but now it becomes a traditional food and has been consumed in family reunions and special occasions (Nuwer, 2014). Furthermore, many Vietnamese think dog meat is a commonly available protein-rich food which is an alternative to the pork, chicken or beef. Report reveals that dog meats are consumed by foreign workers in Malaysia (Nagpal, 2008). Despite of protest from animal welfare group, thousands of dogs are carried out for consumption in Yulin which is dog meat eating festival in China (Figure 2.6 a, b) (Osborne, 2014).

Figure 2.5 a) In South Korea, Traditional markets offer dog meat for consumption (Lee, 2008); b) a popular Cambodian dish made of canine meat (Mee, 2009).

Figure 2.6 a) Dogs are carried for sale in China; b) Campaigners protesting the Yulin Dog Meat Festival in China (Osborne, 2014).

2.11 Importance of Canine Species Detection

2.11.1 Halal issue and illegal trades

The estimated turnover of global halal market is 2.3USD trillion excluding Islamic banking (Salama, 2014). Halal food market represents about 20% of the entire global halal market; whereas the total market value was of 661USD billion in 2010 with increasing trend (Chapter 2, Section 2.6). According to the population growth trend and consumer's purchasing power, the demand of halal foods will be about 70% more by the year 2050 (Chapter 2, Section 2.6). It reflects the higher future demand for halal foods. Over the years, attempts have been made to make higher profit through fraudulent labeling of "Halal" brands (Chapter 2 Section 2.8). Thus processed meats and meat products such as salami (Reilly, 2013), meatloaf (Webb, 2013), cottage pies (Webb, 2013), halal pies and pasties (Crossley, 2013), sausages (Easton, 2013), burger (Reilly, 2013), meatball (Tomlinson, 2013), nuggets (Embiricos, 2013) etc have been specially targeted for adulteration.

Labeling of proper meat products either raw or processed state is very important to ensure fair-trade, health and consumer choices (Chapter 2, Section 2.7). Meat species detection demands more efforts when morphological state of the raw meat is changed via mechanical and thermal process (Montowska & Pospiech, 2010). Quality assurance and accurate labeling of food products boost up consumer trust and promote business (Bonne & Verbeke, 2008). Therefore, many countries of the world like Malaysia, Indonesia and Singapore have regulatory bodies for market surveillance and monitoring Halal food standard. Over the years meat producing countries like Brazil, Australia, New Zealand and China have showed intense interest in Halal certification to promote their products in the Muslim World. Dog meats are used for human consumption in certain countries (Chapter 2, Section 2.10). The availability of stray dogs in certain

countries makes it a potential food adulterant while its descendents are not halal for Muslim consumption (Chapter 2, Section 2.1, 2.4). There is no proven assay for canine species detection from the processed foods. Furthermore, there is no census data for canine population in many countries and stray dogs are available free of prices in many parts of the world. In fact, there no market for dog meats in most parts of the world. Hence, combination above factors has made dog meats as a potential adulterant for common and halal meats or meat products.

2.11.2 Zoonotic threat

Canine species detection in meat or food products is also important for public health concern to stop the spread of various zoonotic and food-borne diseases (Bottero & Dalmasso, 2011). Reports demonstrate the severity of rabies infection in Vietnam due to the illegal dog trading from the neighboring countries like Thailand, Cambodia and Laos (Daugherty, 2014). In Vietnam, restaurants serve different dog meat recipe such as thit cho and its popularity has promoted the illegal smuggling of dogs. Thus it has been evading the normal trading and inspection procedures and acting as a carriers for rabies infection (Nuwer, 2014). In 2008, the salmonella poisoning in South Korea has been blamed for dog meat consumption (Oh & Jackson, 2011). Dogs are also known as reservoirs for many pathogens of parasitic zoonoses, such as toxoplasmosis, ancylostomiasis toxocariasis and giardiasis worldwide (Chen et al., 2012). For example, in Italy dogs are believed to act as a reservoir for vector-borne pathogenic disease caused by L. infantum that affected about 200 people annually (Otranto & Dantas-Torres, 2010). In China, the increasing number of dog population has made it difficult to control the canine parasitic zoonotic diseases such as toxocariasis; ancylostomiasis, trichinellosis, echinococcosis, clonorchiasis, leishmaniasis, toxoplasmosis etc (Chen et al., 2012). The epidemiological study on *Trypanosoma cruzi* infection in householders

demonstrates domestic dogs act as a host three times more than the cats in remotes areas of North-Western Argentina (Gürtler et al., 2007). In the Slovak Republic, consumption of smoked sausages prepared from dog meat infected with T. britovi is the cause for the largest outbreak of this parasitic disease affecting 336 people (Reiterova et al., 2007).

2.11.3 Forensic analysis

Human forensic evidence collected from crime scenes is often integrated with biomaterial of canine and feline origin (Halverson & Basten, 2005). For example, in the United States, 55% of houses own at least one dog. This makes it quite common the availability of canine biological evidences at the crime scenes (Kanthaswamy, Premasuthan, Ng, Satkoski, & Goyal, 2012).

2.12 Current Biomarker Based Species Detection Techniques

People cannot verify the ingredients or species of the process foods using organoleptic test or even after consumption. Hence for authentication of animal species in foods need to be varify with a sensitive but easily performable and reliable scientific method while changes of the physical features occur in the food processing steps. Thus, for determining the species of origin in foods, optic or electronic microscopic analyses techniques are not applicable (Cordella, Moussa, Martel, Sbirrazzuoli, & Lizzani-Cuvelier, 2002). These microscopic analysis methods have the drawback to assign the species from different animal originated components (Ali, Kashif, et al., 2012). Therefore, numerous biomarker based assays using lipid, protein and DNA are proposed for detection of animal species in food or food products (Nakyinsige, Man, & Sazili, 2012). The field of these biomarkers for food authentication and there usefulness and limitations have been represented here.

2.12.1 Lipid-based species detection

2.12.1.1 Lipid based assay

For food animal species detection lipid-based methods are based on analysis of the fatty acids composition such as triacylglycerol (TAG) and 2-monoacylglycerol (2-MAG) (SzabO, FEBel, SugAR, & RomvARi, 2007). Thus the positional distribuation of fatty acid in triacylglycerol (TAG) and 2-monoacylglycerol (2-MAG) may allow us to distingush the origin of the species in certain food products. For example TAGs analysis of certain species like farmed pig, wild boar, red deer, moose, rabbit, and goose showed stored monoenoic and n-6 polyenoic acids with higher chain length and unsaturation (except pig) at *sn*-2 position. Ruminants contain abundance odd-chain fatty with *n*-3/*n*-6 ratio 1, while other species has *n*-6 dominance. Pig restrains lower frequency of n-9 at sn-2 position and lower unsaturated 2-MAG (SzabO et al., 2007).

2.12.1.2 Lipid based assay platforms

For species authentication using fatty acid compositions analyis, Fourier transform infrared spectroscopy (FTIR) combined with multivariate partial least square (PLS) or principal component analysis (PCA) is a usefull method (Rohman, Sismindari, Erwanto, & Che Man, 2011). The FTIR method measures the infrared absorption spectrum of the analytes. The FTIR-spectroscopy can obtain the infrared spectrum of the photoconductivity, absorption, emission or Raman scattering of a gas, solid or liquid samples (Griffiths & De Haseth, 2007). FTIR spectrometer can collect high spectral resolution data over a broad spectral range. The infrared spectrometer consist of four major parts namely, light source, interferometer, sample compartment, and a detector which is connected to a computer for spectrum data analysis (Figure 2.7). From the light source the Infrared energy emits due to the effect of the continuous radiations. The Interferometer combines the interferometer, beam splitter, fixed mirror and a moving

mirror. The incoming infrared beam is divides it into two optical beams by the beam splitter and one beam reflects off the fixed mirror and another one reflects off of the moving mirror. An interference pattern generates by the change in the relative position of moving mirror and the fixed mirror, while the divided reflected two beams convene each other again at the beam splitter. The resulted beam passes through sample and focused on the detector for the final measurement of the signals and sent to the computer. The final analysis is done through the analysis of the different spectrum generated by the computer according to the sample.

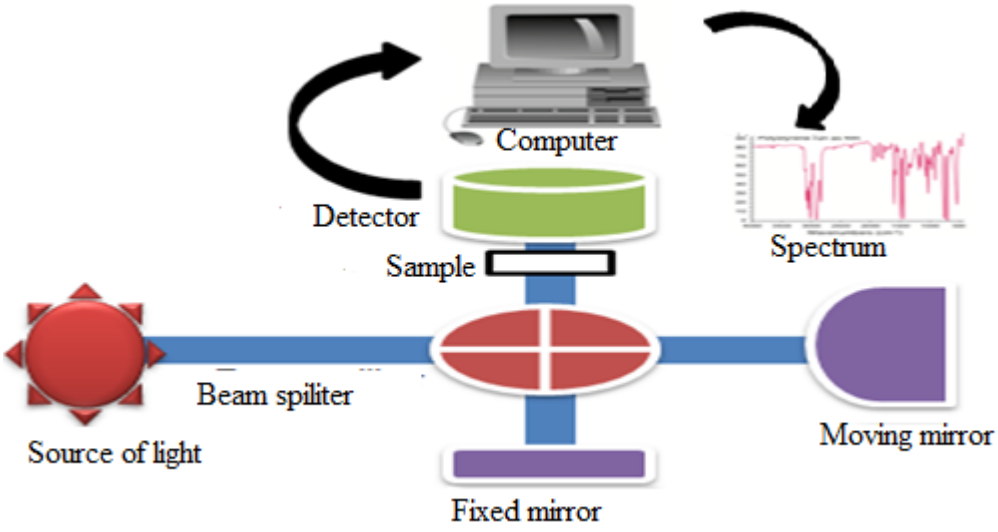

Figure 2.7 Components of a Fourier Transform Infrared (FTIR) spectrometer.

2.12.1.3. Usefulness and Limitation of Lipid based assay

For discrimination of animal and plant originated fats, the fatty acid composition analysis is a constructive marker. Thus, to detect the replacement of vegetable oils with lower costing lard in Halal, Kosher, and vegan food products, fatty acid compositions analysis is useful (Nurjuliana, Che Man, et al., 2011). However, the major draw backs of lipid based assay for the species authentication from meat or meat products is the alteration of of the positional distribution of fatty acid contents and varieties of TAGs and MAGs during cooking process (Ali et al., 2012). Hence, for determination of

animal species inprocessed food products, the lipid-based recognition tools have narrower application.

2.12.2 Volatile Organic Compounds based detection

2.12.2.1 Volatile organic compounds based assay

Volatile organic compounds (VOCs) are organic chemicals originated from the thermal or biodegradation products of amino acids, fatty acids or sugars content of a particular food item (Acevedo et al., 2012). Low boiling points and high vapor pressure can develop a characteristic aroma profiles and pile up in the headspace of a food material which can be used for identity analysis a distinct food samples.

2.12.2.2 Volatile organic compounds based assay platform

Gas chromatography-mass spectrometry (GC-MS) is a handy analytical method that can perform both separation and subsequent identification of different analytes from solid and liquid food samples. GC-MS combines the features of the gas-chromatography and the mass spectrometry (Figure 2.8). It determines the different substances present in the sample. The gas chromatograph is based on capillary column with different dimensions (diameter, length, film thickness) and phase properties (e.g. 5% phenyl polysiloxane). The separation of the different molecules occurs according to the chemical properties of the samples and their relative affinity for the stationary phase of the column. The mass spectrometer breaks each of the molecules into ionized fragments and detects these fragments by means of their mass-to-charge ratio. Combining these two techniques reduces the possibility of error, as it is improbable to have similar pattern of two different molecules in a sample in both gas chromatograph and mass spectrometer. GC-MS requires extraction and pre-

concentration of the VOCs to be analyzed. To overcome this limitation and to reduce the analytical steps with higher efficiency, solid-phase micro-extraction (SPME) is an updated analytical technique (Balasubramanian & Panigrahi, 2011). The basic principle of the SPME is the exposure of a pre-coated polymeric surface with the target sample and analysis of the transmission of the extracted compound with a sensor couple with GC and other analytical tool. The SPME coating is based on the sample target along with the polarity and volatility attributes of samples.

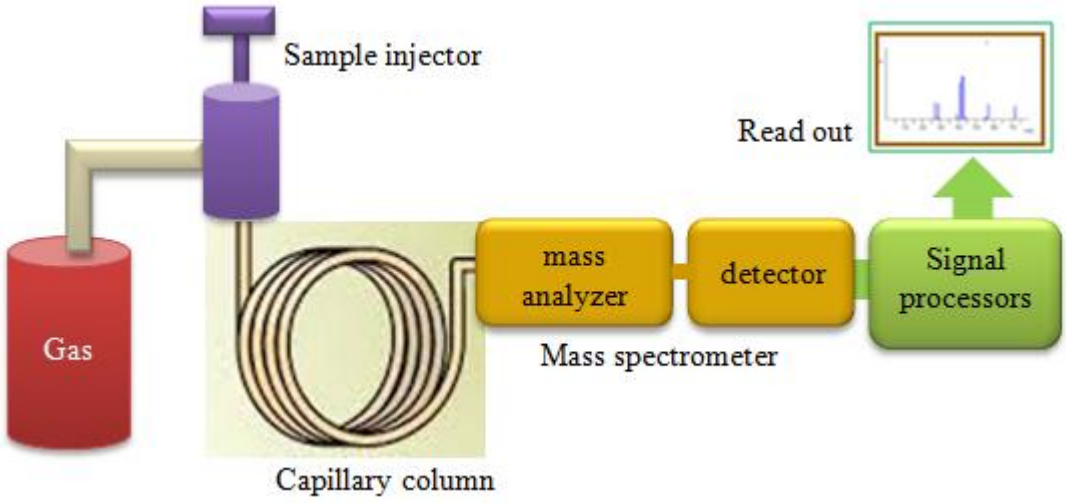

Figure 2.8 Schematic of GC-MS

The recent electronic nose or E-nose can detect the volatile compounds present in the headspace of a food sample by an array of semi-selective gas sensors. It is based on the device to detect odors or flavors similar to human olfaction and composed of a sensor array, preprocessor unit, and a pattern recognition system (Figure 2.9). Thus E- nose system consists of a multisensor array, an artificial neural network and software to analyze the digital pattern-recognition algorithms, and reference-library databases (Wilson & Baietto, 2009). Its instrumentation requires three major units for sample handling, detection and data processing. It is based on the array platform with non-

specific sensor and a variety of memory response to the different odors of biological samples. Thus, the specific odor generates a pattern or fingerprint to recognize an unknown odor form memory response of the sensory array (Peris & Escuder-Gilabert, 2009). Another interesting method for food analysis is sniffing the gas chromatographic effluent of a representative isolate of volatile compounds. It associates the odor activity with the eluting compounds of the food compounds (Van Ruth, 2001).

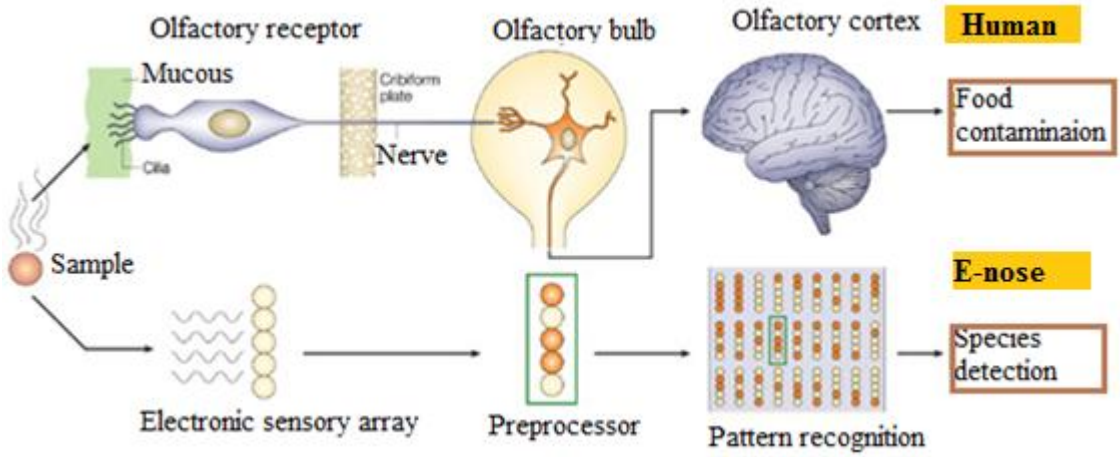

Figure 2.9 The electronic devices simulate the different stages of the human olfactory system and volatile odor recognition using E-nose for organism species detection, adapted from (Turner & Magan, 2004).

2.12.2.3 Usefulness and limitation volatile organic compounds based assay

GC-MS is a potent and sensitive method for simultaneous separation and recognition of solid or liquid volatile analysts. However, most of the analytes from different meats and meat products identified by GC-MS are similar in nature which needs to discriminate by using statistical tools. Furthermore, it is an expensive and time-consuming procedure in comparison to e-nose (Nurjuliana, Che Man, et al., 2011; Nurjuliana, Man, et al., 2011). GC-MS based analysis needs experts and well-trained personnel while e-nose has progressed with easily operative analytical tool (Peris &

Escuder-Gilabert, 2009). E- nose technology can be performed by using relatively small amount of sample with faster analysis, however, this technique have the drawback of less selective sensors for particular types of compounds. Thus the non specific identification or quantization of individual compounds may misled the result of adulteration (Reid, O'Donnell, & Downey, 2006). GC-sniffing is an interesting technique for analysis of volatile compounds of foods but the effectiveness of it to distinguish different species in foods still in question.

2.12.3 Protein Based Species Detection

2.12.3.1 Protein based assay

In certain literatures for animal species authentication the usefullness of species specific protein analysis which are described below.

a. ***Quantification of Histidine Dipeptides***

Certain histidine dipeptides such as anserine (ß-alanyl-L-1 methylhistidine) Carnosine (ß-alanyl-Lhistidine) (CAR), (ANS), and balenine (ß-alanyl-L-3 methylhistidine) (BAL)) are only present in animal tissues, such as muscle, kidney, liver, and heart and can distinguish animal originated products from plants (Aristoy & Toldrá, 2004). These naturally occurring molecules of animal tissue with strong sensory attribute can generate specific meat flavor (Aristoy & Toldrá, 2004). Dipeptide compounds are also species specific, thus, the determination of the relative abundance of carnosine and anserine (CAR/ANS) in feeds can be used to trace out the animal's source of feed proteins. For example, CAR/ANS ratio in pork, beef, lamb, and poultry is 17.88±3.74, 8.08±1.91, 0.95±0.26, and 0.20±0.08, respectively (Aristoy et al.2004). Analysis of dipeptides can easily resolve the presence of animal proteins and they are

not found in plant and plant products. CAR/ANS ratio≥0.5 strongly suggests the presence of mammalian proteins in feeds (Aristoy & Toldrá, 2004; Schönherr, 2002).

b. Analysis of muscle proteins

The origin of species can be determined by the analysis of muscle proteins such as sarcoplasm (cytoplasm of the muscle cell) or sarcomere (structural and functional units of muscle fiber). Parvalbumins are a family of heat-stable, acidic, calcium-binding proteins account for a major part of sarcoplasm of fish muscle. The isoelectric pH under native conditions varies between 3.8 and 5.3 (Addis et al., 2010; Berrini, Tepedino, Borromeo, & Secchi, 2006). Thus, the iso-electric profiling of sarcoplasmic proteins, such as parvalbumins can be used to differentiate certain fish species (Berrini et al., 2006). Two-dimensional electrophoresis (2-DE) can complete the identification when isoelectric focusing shown identical pattern but unable to discriminate closely related species. Furthermore, 2DE map of sarcomeric proteins, such as myosin light chain (MLC), provides information for the species, freshness and storage condition of the muscle tissues (Martinez & Jakobsen Friis, 2004; Pischetsrieder & Baeuerlein, 2009). Thus combination of electrophoretic technique with certain proteomic tools such as in-gel digestion and mass spectrometry were also described species-specific MLC analysis from muscular tissues mixtures of different species (Martinez & Jakobsen Friis, 2004; Pischetsrieder & Baeuerlein, 2009).

c. Analysis of species-specific osteocalcin

Analysis of Species-Specific Osteocalcin (γ-carboxyglutamate containing protein) can also be used for species authentication. The Osteocalcin (OC) which is a universal bone constituent contains 49-amino acid of calcium binding protein produced by osteoblast. It constitutes about 10–20% of non-collagen proteins in bone. Although

the core of this protein is conserved in evolution, but its N terminus contains polymorphic information for species differentiation. This small size, secure and compact molecules are comparatively stable (Balizs et al., 2011).

d. Detection of species-specific proteins by ELISA

ELISA is an Immunological technique (Asensio, González, García, & Martin, 2008), it immobilizes either an antigen or antibody onto a solid surface along with the detection of antigen–antibody interactions. It works with a labelled enzyme that converts substrate into a color product or releases an ion which reacts with another reactant to create a detectable change in color (Bonwick & Smith, 2004). Indirect and sandwich ELISA are the two most common forms of ELISA used in food analysis. Indirect ELISA is based on two antibodies, one binds to a specific antigen and another one to an enzyme that converts a substrate to a colored product (Hsieh, Zhang, Chen, & Sheu, 2002). In the sandwich ELISA, the antigen is bound between the two antibodies such as capture antibody and detection antibody (Liu, Chen, Dorsey, & Hsieh, 2006). The antibody is bind to an enzyme creating detectable color change. ELISA can be used both qualitative or quantitative analysis such as qualitative ELISA can provide positive or negative results and the quantitative ELISA can measure the concentration form a standard curve generated by a serial dilution of the targets (Asensio et al., 2008). Furthermore, for the detection of denatured proteins, polyclonal antibodies (PAbs) can provide a broad recognition tool with different epitopes (Asensio et al. 2008).

2.12.3.2 Protein based assay platform

Proteins molecules are composed of varying charges and complex shapes and may not fully or even partially migrate into the polyacrylamide gel when placing a negative to positive electromotive force (EMF). Hence, proteins are

usually denatured in the presence of detergent such as sodium dodecyl sulfate (SDS) and it coats the proteins with a negative charge.[3] As the denatured protein act like long rods instead of having a complex tertiary shape it can be migrate through the gel. Thus the most commonly proteins molecules are usually analyzed by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2.10). It can also be analysed by native gel electrophoresis, by quantitative preparative native continuous polyacrylamide gel electrophoresis (QPNC-PAGE), or by 2-D electrophoresis.

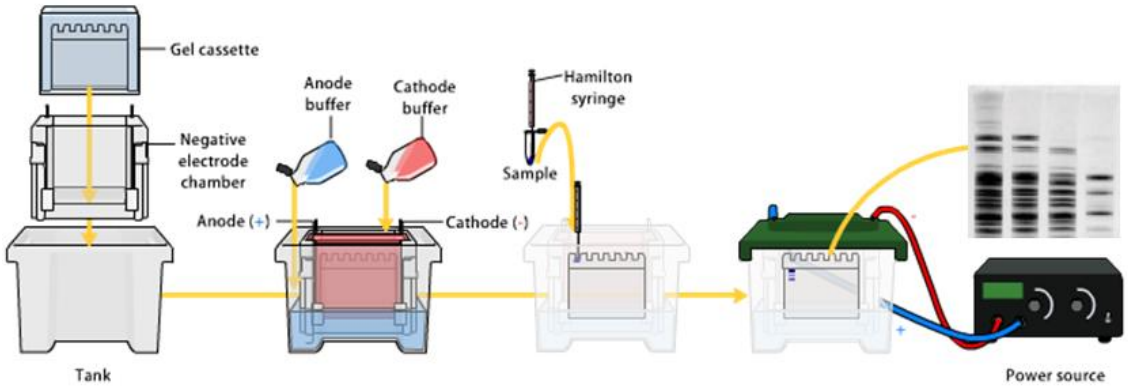

Figure 2.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis for protein based species detection.

Liquid chromatography–mass spectrometry (LC-MS) or High performance liquid chromatography-mass spectrometry (HPLC-MS) are analytical chemistry tools those combines the physical separation capabilities of liquid chromatography (LC) or High performance liquid chromatography (or HPLC) with the mass analysis abilities of mass spectrometry (MS) and can be utilized for protein analysis. A typical HPLC instrument composed of a sampler, pumps, and a detector. The sampler carries the sample mixture into mobile phase stream which subsequently brings the sample into the column. The required flow and composition of the mobile phase is delivered by the pumps. Finally the signals are generated by the detector from the sample component passing through the column. A digital microprocessor is attached with the HPLC instrument along with software for analysis of the data. The HPLS signal from the

detector is proportional to the amount of sample component and thus it allows quantitative analysis of the sample components. The mass spectrometry does the ionizing of chemical compounds for generating charged molecules or molecular fragments and measuring their mass-to-charge ratios.

For analysis of ELISA assays for specific protein, absorbance detection can be performed by plate a reader which is also known as microplate readers or microplate photometers. A specific light source illuminates the sample using a wavelength which is selected by optical filter, or a monochromator from one side. A light detector placed on the other side of the well quantifies the amount of the initial (100%) light that was transmitted through the sample. The concentration of the target molecule will typically be related with the amount of transmitted light. The most commonly used microplate format in ac9ademic research laboratories or clinical diagnostic laboratories is 96-well (8 by 12 matrix) with reaction volume 100 to 200 µL per well. Higher density micro plates of 384 or 1536 well with 5 and 50 µL sample per well are used for the screening of number of samples.

2.12.3.3 Usefulness and limitation of protein based assays

OC in MBM can be detected and quantified by highly sensitive MALDI-TOF and Q-TOF mass spectrometry (MS), but MS is costly and needs specialized skills to operate and interpret data (Ali et al., 2012). Furthermore, electrophoretic and proteomics tools are laborious, expensive, specialized skills needed, and also not suitable to analyze complex mixtures (Addis et al., 2010; Ali et al., 2012; Pischetsrieder & Baeuerlein, 2009). MAbs and PAbs can be used for animal species detection from soluble and structural proteins of the muscle cells. However, protein-based techniques are limited when assaying heat-treated material due to denaturation of soluble proteins

during food processing (Fajardo et al., 2010). ELISA is a useful technique for discrimination of tissue source and type of animal proteins, but cross-species reaction between closely related species have been reported in few cases. Furthermore, the sensitivity of ELISA assays under complex pool of multiple species is not constant (Liu et al., 2006) and not equivalent to the DNA-based methods assay (Fajardo, González, Rojas, García, & Martín, 2010).

2.12.4 DNA Based Species Detection

Recent DNA based analytical platform showed huge potentiality to food manufactures, researchers, and regulators for qualitaive and quantitave analysis of processed food ingredients. In finished commercial products to identify the declared or undeclared ingredients, DNA-based methods have splendid effeciency to illuminate a minute level of adulteration in process foods. The key features for specific DNA molecule detection is the hybridization of complimentary sequence. The interaction of A–T and G–C bases between two DNA strands occur through the hydrogen bonds and π–π stacking. This hybridization process with its exquisite fidelity in molecular recognition makes DNA a versatile, programmable and "smart" polymer. This specific interaction of A–T and G–C with canonical form of base pairing is also called Watson–Crick base pairing. The hybridization features of a stable helical DNA structure can thermally be "melted" where the dsDNA native strands can form ssDNA dissociated by heating above a critical melting temperature (T_m). Using these features of complimentary DNA base hybridization denaturation of dsDNA primers can be designed to detect specific DNA target. Furthermore, DNA molecule is also inherently directional containing a 5′-terminus (upstream, 5th carbon position on the ribose) and a 3′-terminus (downstream, 3rd carbon position on the ribose) which chemically encode the orientation of a DNA molecule. A phosphate group at the 5′-end and a hydroxyl

group at the 3′-end make the directional formation of a dsDNA, wherein the 5′-end from one DNA strand or primer may align with the 3′-end of from another strand. The DNA bases can also form a non-covalent bonds within the chain through π–π stacking, hydrogen bonding, hydrophobic interactions, and van der Waals interactions which may result in the formation of DNA secondary structural motifs, such as the G-quadruplex and the i-motif (C-rich strand). This feature of secondary structures formation of DNA can be used for certain functional utility such as sensing applications in molecular beacons or aptamers.

2.12.4.1 Nanotechnology based detection

2.12.4.1.1 Nanomaterials

Nanomaterials describe the materials with a single unit size (in at least one dimension) between 1 and 1000 nanometers (10^{-9} meter). Nanoscientists have ascertained the characteristic of certain nanomaterials with nanoscale dimension of 1–100 nm with increased surface to volume ratio for specific application (Chithrani, Ghazani, & Chan, 2006). For example, Gold nanomaterials (AuNMs) with unique optical properties and highly resonant particle plasmons can be synthesized at large size range (1-100 nm) and shape (1:1-1:5 aspect ratio) for biomolecule detection (Chithrani et al., 2006). Simple conjugation and upholding capability with different biomolecule such as DNA offer certain nanomaterial to act as an ideal transducers (Cao, Ye, & Liu, 2011; Taton, Mirkin, & Letsinger, 2000; Zeng et al., 2011) for species authentication platform.

2.12.4.1.2 Nano-platform

Latest nano-platform come forward with attractive nonmaterial with excellent physio-chemical properties to create building block for DNA molecule reorganization. The key component of the AuNMs in probe hybridization detection is the surface plasmon absorption on distance-dependent surface (Wang, Yang, Yang, & Tan, 2009). Silica nanomaterials with exclusive geometric properties in conjugation with various hybrid nanomaterials may offer a highly stable nano-platform with increased sensitivity. For example, dye-doped fluorescent silica nanomaterials may facilitate increase signal amplification (Wang et al., 2006) and magnetic silica nanoparticles assist molecular loading and transportation (Santra et al., 2001; Trewyn, Slowing, Giri, Chen, & Lin, 2007). Sandwich silica based DNA detection assay composed of a captured DNA immobilized on a glass surface; a probe sequence attached to TMR-doped silica nanoparticles; and the unlabeled target sequence complementary to both the capture sequence and probe sequence (Zhao, Tapec-Dytioco, & Tan, 2003). Carbon nanotubes (CNTs) are imperative nanomaterials with cylindrical structure with optical and mechanical properties for DNA detection (Prato, Kostarelos, & Bianco, 2007). They are basically seamless rolled up graphene sheets of carbon and divided into single-walled nanotubes (SWNTs) and multi-walled nanotubes (MWNTs). The effective mechanism of specific DNA target detection is based on a non-covalent attachment with the side wall of SWNTs by π-π interaction. Fluorescence signal amplification properties of SWNTs with specific DNA target in aqueous medium (Jeng, Moll, Roy, Gastala, & Strano, 2006) made them very suitable for electrochemical detection (Wang, Kawde, & Musameh, 2003). Quantum Dots (QDs) are inorganic semi-conductor nanocrystals with size-dependent stable luminescence properties with high quantum yields, broad absorbance bands, but narrow emission spectra and high photochemical stability against

photo-bleaching (Ho, Kung, Yang, & Wang, 2005). QDs have distinct photophysical properties for DNA analysis (Wang et al., 2009).

2.12.4.1.3 Usefulness and limitation of nanotechnology

Ample analysis of process food in the last decades imitated the usefullness of DNA as the most stable biomolecule for animal species authenticaion for raw as well as comercial products for animal-derived materials. Different nanoplatform received great attention globally due to the ability to minimize cost and time. However, nanotechnology approaches to detect DNA for species authentication in foods are still immature or in conceptual advancement (Ali et al., 2012).

2.12.4.2 Polymerase Chain Reaction (PCR) Based Assay

2.12.4.2.1 Polymerase Chain Reaction (PCR) Assay

Polymerase Chain Reaction (PCR) (Mullis & Faloona, 1987) is an in vitro amplification procedure from a specific DNA template to large quantities from complex pool of DNA using a simple enzymatic reaction. For specific DNA detection with simple PCR assay, oligonucleotide primers are designed targeting the complementary sequence at the ends. Admixture of the primers with DNA template, deoxyribonucleotides and suitable buffer followed by heat treatment denature the original strands. Further cooling promotes primer annealing and by the action of DNA polymerase new strands are synthesized. Furthermore, new copies of DNA are formed by repeated cycle of denaturation, annealing, and polymerase action. Finally an exponential increase in the total number of specific target DNA occurs with a theoretical abundance of 2n, where n is the number of cycles (Figure 2.11) (Erlich, Gelfand, & Sninsky, 1991). The use of different PCR based assays such as PCR

products sequencing, DNA microarray, Species Specific PCR, PCR-RFLP and Real Time PCR are described below in individual headings.

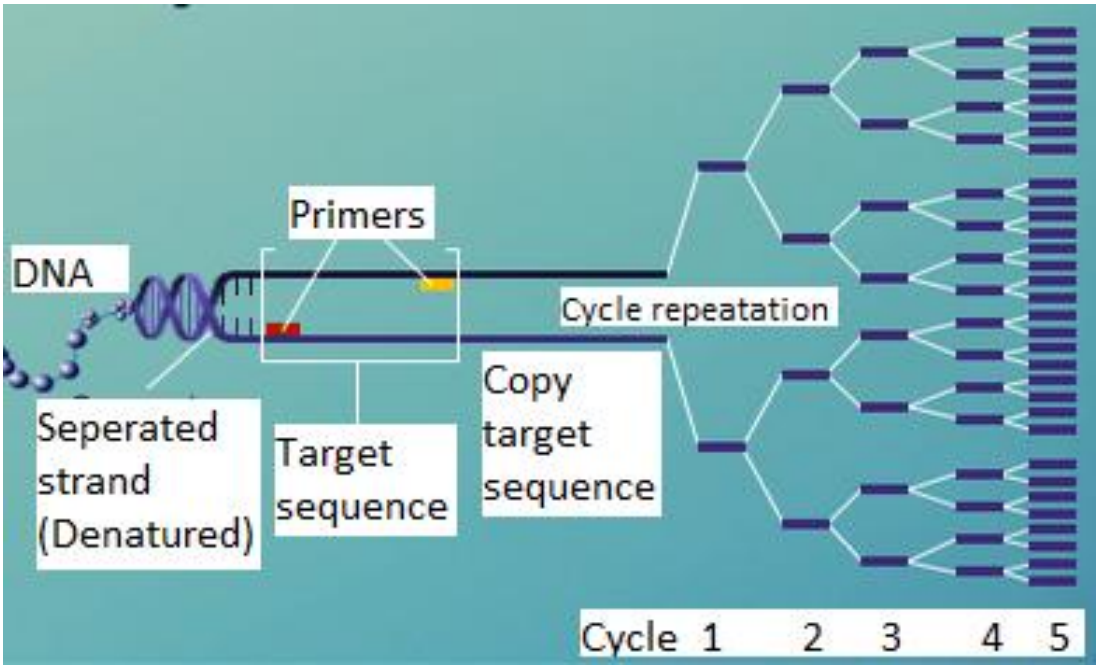

Figure 2.11 Specific DNA target replication by PCR.

2.12.4.2.2 PCR assay platform

For PCR assay the thermal cycler may compose of four basic units such as multiple plate count for samples, a circuit board, thermal-base layer and cooling elements (Figure 2.12). The efficiency of the PCR assays is based on the rapid and accurate maintenance of the reaction temperatures of thermal-cycling system. The circuit board maintain the heating and cooling of the thermal cycler and can be achieved using different elements such as Peltier. Thermal-Base unit incorporation beneath the circuit can ensure the optimal heat distribution across the sample plate. It also optimized the well-to-well homogeneity and maximized inter-well assay reproducibility. The cooling system below the Thermal-Base unit maximizes the inner surface area for rapid heat absorption

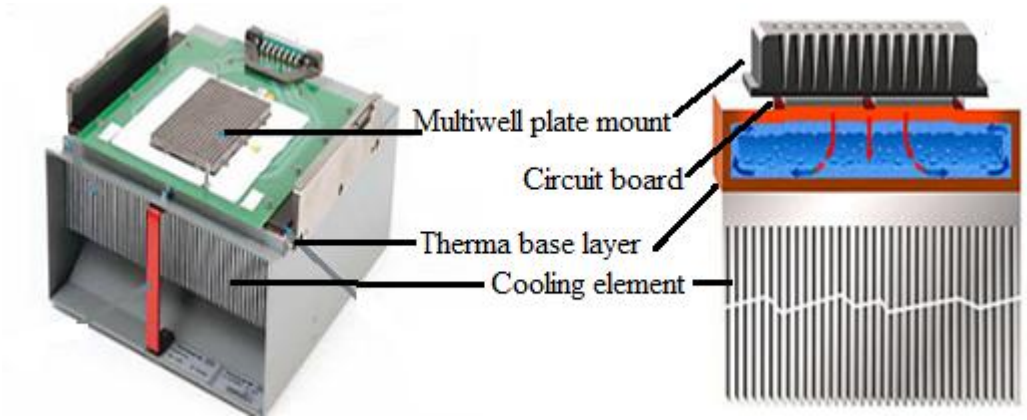

Figure 2.12 PCR thermal cycler

2.12.4.2.3 Usefulness and limitation of PCR assay

a. Easy and faster: Cloning of DNA cloning by PCR around 30 cycles containing a denaturation, synthesis and reannealing step can be performed using relatively short amount of time than other methods. It is easy to set up a PCR reaction with thermal instrumentation facilitated by gradient PCR machine. Furthermore, the recent PCR assays forwarded with development of computer based primer designing tools and oligonucleotide synthesis by commercial or academic bodies (Erlich et al., 1991).

b. Highly Sensitive: PCR is able of amplify specific DNA target from minute amounts of DNA even from a single cell (Li et al., 1988). Such superb sensitivity opened up the opportunity for utilization of PCR for Halal authentication from trace amount DNA from highly process food even up to 0.01 % of non halal adulteration.

c. Robustness: The PCR assay can be performed from a range of nucleic acid sources. The suitable templates for PCR amplification may vary from purified DNAs from various species to specific sequences from material in which the DNA is badly degraded or embedded in a medium from which conventional DNA isolation is problematic. Therefore, PCR platform can recover and amplify the species specific DNA target from a few copies to easily detectable quantities, even from a highly degraded samples and complex pool of DNAs (Ali et al., 2012).

There are some limitations of PCR assay which are given below.

a. Because of the high sensitivity of the PCR assay, any form of contaminant in the sample even in trace amounts of DNA may produce misleading results (Smith & Osborn, 2009).

b. For designing the primer for PCR assay, it needs prior information of the specific sequences. Hence, it is only applicable for the species which have the necessary sequence information (Garibyan & Avashia, 2013).

c. The primers designed for the PCR assay may anneal nonspecifically to the non target similar sequences but not completely identical, to target DNA (Garibyan & Avashia, 2013).

2.12.4.3 Real-time polymerase chain reaction

A Real-Time Polymerase Chain Reaction (RT-CPR) or Quantitative PCR (qPCR) is a molecular laboratory technique based on polymerase chain reaction (PCR) assay which amplify the specific DNA target. The assay allows a quantitative or real time monitoring of the amount of DNA amplified in each cycle either by using fluorescent dyes or fluorescently-tagged oligonucleotide probes (Higuchi, Dollinger, Walsh, & Griffith, 1992). Thus it simultaneously detects or quantifies a targeted DNA molecule. The real time PCR result can be achieved faster with higher consistency than conventional PCR, due to perceptive fluorescent chemistry and exclusion of the post-PCR end point detection system.

2.12.4.3.1 Fluorescence measurement

The fluorescence intensity during the real time PCR analysis is correlated to the quantity of DNA template (Wong & Medrano, 2005). Depending upon the concentration of the DNA, after few cycles of the real time run, fluorescence intensity exceeds a threshold level. Thus, the fluorescence signal exponentially increases

subsequently as the DNA templates amplified and reach above the background level. However it eventually reaches a plateau phase due to the saturation of the fluorescence (Figure 2.13). The saturation detection of fluorescence by the real time PCR platform is not related to the starting copy number of the DNA template.

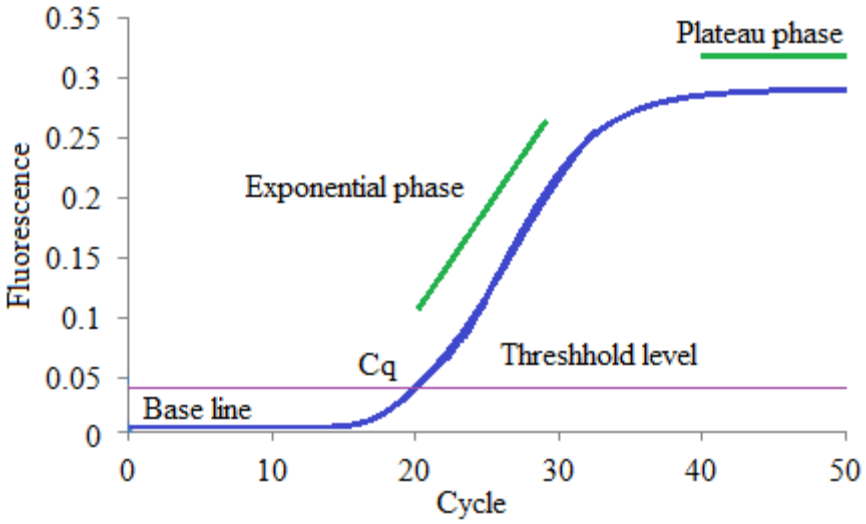

Figure 2.13 Phase of the fluorescence signal in Real time PCR.

2.12.4.3.2 RT-PCR fluorescence detection

For detection of the fluorescent signal in RT-PCR two types of chemistries are utilized namely SYBR-based chemistry and probe-based chemistry.

SYBR-based chemistries are useful for the detection and quantification of the double stranded DNA (dsDNA) molecules. The signal amplification is based on the intercalating fluorphores or fluorescent dyes that bind within the dsDNA (Figure 2.14). However, the most commonly used fluorophore dye such as SYBR Green may bind to all of the dsDNA in the samples including nonspecific PCR products such as Primer dimer. The other fluorophores used for RT-PCR assay include EvaGreen™, BEBO, SYTO9 and BOXTO.

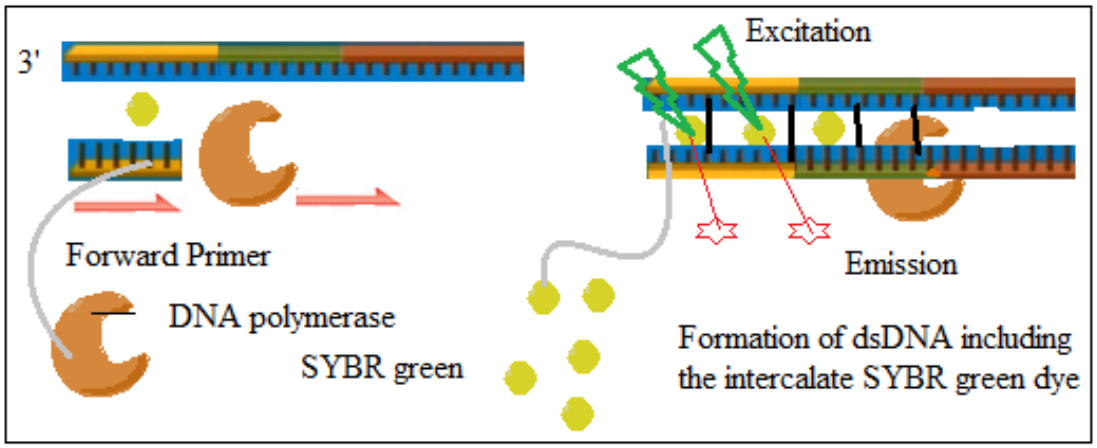

Figure 2.14 Fluorescent signal amplification using SYBR Green Chemistry. Primers attached with complimentary target DNA and SYBR Green molecule binds with the dsDNA. Elongation of the target by DNA by polymerase enzyme leads the excitation and emission of the fluorescent signal.

For the detection and quantification specific target probe-based chemistries are based on the oligonucleotide probes tagged with fluorescent molecule. A covalently bonded fluorescent dye and a quencher molecule are attached at the 5'and 3' end of the probe sequence. The Fluorescent reporter probes only bind with the complimentarysequence of samples, and hence it significantly increases the assay specificity. The entire probe sequence with the reporter and quencher dye at the proximity consent to Fluorescence Resonance Energy Transfer (FRET) and stop the emission of the fluorescent. For real time PCR assay various reporter dyes such as 6-carboxyfluorescein (FAM), tetracholro-6-carboxyfluorescein (TET), hexachloro-6-carboxyfluorescein and quencher such as 6-carboxytetramethyl-rodamine (TAMRA) or 4-(dimethylaminoazo) benzene-4-carboxylic acid (DABCYL) are used. In the PCR amplification process the probe anneal with the specific target and elongation of the target sequence occurred by the Taq polymerase enzyme. During polymerization, it cleaves the probe and allows for an increase fluorescent emission (Figure 2.15). The most common used specific probe is a TaqMan probe (hydrolysis probe), others include

Molecular Beacons (a hairpin probe), hybridization probes, LightUp® probes and Solaris (probe with minor groove binding moeity and superbase).

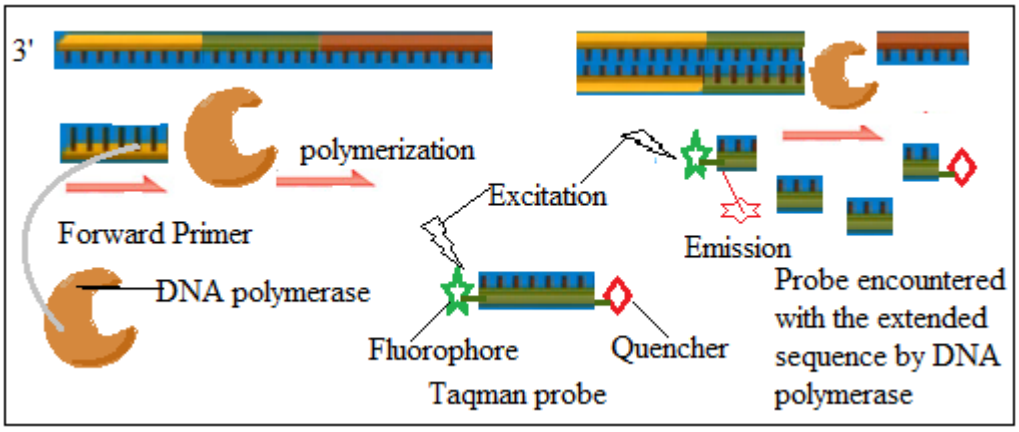

Figure 2.15 Probe based Chemistry (Taqman probe) in RT-PCR. The primer is extended by the DNA polymerase and encounters the probe leading no longer quenching of the flurophore and increase emission of the fluorescence along with the specific target detection.

2.12.4.3.3 Real time quantification methods

Quantitative analysis of the real time PCR result can be done by following two methods, absolute quantification and relative quantification. In absolute quantification, calculation of the unknown sample quantity is based on the analysis of Ct value and development of a standard curve. Usually, the standard curve is generated using known samples from plasmid with a serial dilution. However, recently the synthetic single stranded oligonucleotide is used to develop the standard curve. It is less prone to the bias quantification using a spectrophotometer. Furthermore, the simple PCR amplification process simplifies the generation of standard curve using relatively pure oligonucleotide. On the other hand, the relative quantification is based on the mathematical equation. It calculates the gene expression of a specific target relative to another reference sample or calibrator such as an untreated control sample or RNA from normal tissue.

2.12.4.3.4 Usefulness and limitation of RT-PCR

Advancement of the RT-PCR assays coupled with high-throughput and automated technology (Klein, 2002). RT-PCR assay combines the detection of target DNA molecule with quantification which is measured by florescent signal amplification during each PCR cycle. Generation of standard curve using the RT-PCR value (Ct value) with a known concentration of DNA may allow us to estimate concentration of a unknown sample (Ali et al., 2012). Thus, it allows us to determine the food adulteration in quantitative format (Ali, Hashim, et al., 2012). RT-PCR assays coincide with a wide dynamic range of quantification of 7–8 logarithmic decades along with high technical sensitivity (<5 copies) and higher precision (<2% standard deviation) (Klein, 2002). Therefore, RT-PCR assays with a detection probability from minute traces of different animal species from a complex pool of food products are considered as a promising molecular identification tools for meat species authentication (Köppel, Zimmerli, & Breitenmoser, 2009).

Although RT-PCR assay is capable of providing quantitative results by real-time monitoring of the accumulating PCR product, it requires high costing specifically designed instrument and reagents along with an appropriately designed probe (Ali, Hashim, et al., 2012; Yusop, Mustafa, Che Man, Omar, & Mokhtar, 2012). Furthermore, the higher sensitivity of the assay and shorter amplicon target it may lead to cross-species amplification in heterogeneous background (Ali, Hashim, et al., 2012).

2.13 Different DNA Based PCR Assays for Species Detection

2.13.1 PCR Sequencing

2.13.1.1 PCR Sequencing Assay

Single banded PCR product using a universal primer can be obtain from a varirty of animals (Karlsson & Holmlund, 2007). Subsequent sequencing of the PCR products can descreminate the animal species/plant species in the food products (Fajardo et al., 2010). Thus for the species authentication by PCR sequenecing, mitochondrial cytochrome b, 16S rRNA and 12S genes are mostly used genetic markers dut to the avaliability of the sequence information in the public databses and presence of adequate polymorphism (Karlsson & Holmlund, 2007). However some of the report have made for the use PCR sequencing of nuclear 18S rRNA or the diglyceride acyltransferase 1 to detect animal species such as buffalo, kangaroo or crocodile (Fajardo et al., 2010; Venkatachalapathy, Sharma, Sukla, & Bhattacharya, 2008). Recent development of DNA barcoding is a simple but powerful toll for differentiation of animal or plant species on the basis of sequence diversity in a short, standardized genomic region. PCR amplification and sequencing of a standard fragment of 650 bp mitochondrial cytochrome oxidase I (COI) gene produces reference sequences or "DNA barcodes". Using this molecular identification tag >95% of species can be distinguished from the test assemblages of different animal groups (Fajardo et al., 2010; Ferri, Alu, Corradini, Licata, & Beduschi, 2009).

2.13.1.2 Usefulness and limitation PCR sequencing

Sequencing of the amplified PCR products may provide the highest amount genetic information to distinguish the animal species without using any enzymes or post-analysis (Fajardo et al., 2010). However sequencing is an expensive method and it

need specialized instrumentation. Hence it is not useful for routine analysis. Furthermore, sequencing of "DNA barcodes" with a longer amplicon based PCR product may breakdown during the food processing leading the false negative result (Ali, Hashim, et al., 2012).

2.13.2 PCR-RAPD

2.13.2.1 PCR-RAPD assay

The random amplified polymorphic DNA (RAPD) methods have been used for the identification of different species such as meat animal, fish and vegetable (Arslan, Ilhak, Calicioglu, & Karahan, 2005; Mohindra et al., 2007). The method utilized a short arbitrary primer which ties at the random location of the genomes and amplified by PCR, yielding a specific pattern for each animal species. Thus, the specific species is determined by DNA fingerprints obtained from separation of different sized amplified fragments using gel electrophoresis (Fajardo et al., 2010).

2.13.2.2 Usefulness and limitation PCR-RAPD assay

PCR-RAPD analysis with arbitrary primers provides the opportunity that it does not require any specific gene sequence information to create a species-specific patterns. It is a simple tool and does not require multiple steps of DNA restriction or sequencing (Wu, Liu, & Jiang, 2006). However, the major disadvantage of PCR-RAPD is the reproducibility. Furthermore, it is not suitable for admixed sample analysis and requirement of high quality DNA for PCR initiation for RAPD profiling restricted the utilization of it highly processed meats or food product analysis (Fajardo et al., 2010).

2.13.3 Oligonucleotide Microarray

2.13.3.1 Oligonucleotide Microarray based assay

DNA biochip (DNA microarray) is one of the latest technologies which can analyze complex mixtures of PCR products to identify different species in single assay run (Teletchea, 2009; Teletchea, Bernillon, Duffraisse, Laudet, & Hänni, 2008). It is based on the amplification of a small fragment of mitochondrial gene target from conserved sequence of closely related species using pair of primers and labeling with fluorescent dye (Cy3/Cy5). Although the amplification procedure contains consensus sequences at the two termini, however it carries adequate interspecies variation information in the intermediate region. Thus, the species-specific short probes are developed with a functional group (NH_2/COOH) at one end to facilitate the immobilization while another end labeled with a fluorescent dye (Cy3/Cy5) allow the species specific detection.

2.13.3.2 Usefulness and limitation of oligonucleotide microarray

Microarray technology widen up the opportunity to detect multiple species. However, the reliability of this method is reduced due to the cross-species identification from closely related species. Furthermore, the sensitivity (0.5 %w/w) of the microarray technology is lower than the Real Time PCR assay (Iwobi, Huber, Hauner, Miller, & Busch, 2011). It cannot provide quantitative information which is required to draw up a boundary between permissible and non-permissible limits of contamination and/or intentional and accidental level of adulteration of certain species in processed foods (Ali et al., 2012).

2.13.4 Species specific PCR

2.13.4.1 Species specific PCR (SSP) assay

Species specific PCR assay allow us for direct detection of the species in food products by using specially designed oligonucleotide primers. The primers facilitate the identification of specific target sequence from a complex pool DNA under restrictive PCR conditions. Most of the documented species specific PCR assay were based on multicopy mitochondrial (mt) genes target (Ali et al., 2012) which may boost the availability of target DNA template even form a highly degraded samples such as the process foods. The PCR end point detection may be performed by different gel electrophoresis techniques such as agarose or capillary electrophoresis. Thus, for species authentication using species specific PCR assay, both the singleplex (İlhak & Arslan, 2007; B. Mane, Mendiratta, & Tiwari, 2012) and multiplex (María Rojas et al., 2009; Sakai et al., 2011; Yang, Kim, Byun, & Park, 2005) assays have been reported.

2.13.4.2 Usefulness and limitation of SSP assay

SSP assay using well defined species-specific primers may amplify the target sequences and define the origin of the species from complex background of different DNA admixed without subsequent sequencing or restriction fragment length polymorphism (RFLP). Hence, it is a useful biological marker and well adapted assay for routine analysis of raw or processed food samples (María Rojas et al., 2009). Furthermore, PCR assay using short amplicon based species-specific primers offers a simple but specific and high sensitivity species authentication platform for highly degraded or thermally treated food samples (Fajardo et al., 2010; Rojas et al., 2011). However, the downside of this specific PCR assay is that it relies on the need of accurate data on the species target sequences in order to design the corresponding

specific primers (Rojas et al., 2009). Multiplex PCR assays techniques over the singleplex assay may facilitate multiple species detection from single and reduce cost and time. However, the intrinsic complexity, inconsistent sensitivity for different-length DNA templates, low and asymmetrical amplification efficiency have reduces their application for target quantification (Ali et al., 2012; Iwobi et al., 2011). Alternative singleplex PCR assays is proven for more accuracy, sensitivity and robustness, even it cannot detect multiple species at single assay run (Fajardo et al., 2010; Yusop, Mustafa, Che Man, Omar, & Mokhtar, 2012).

2.13.5 PCR- Restriction fragment length polymorphism

2.13.5.1 PCR- Restriction fragment length polymorphism assay

PCR-restriction fragment length polymorphism (RFLP)-based assay which is also known as cleaved amplified polymorphic sequence (CAPS) analysis, is one of the most used laboratory tool to study genetic variation. The Restriction endonuclease enzyme cut the DNA sequences into pieces according to the organism type or species or state, with a minor difference in the specific homologous site. Thus, the DNA sample yield specific fragmentation pattern after break down by restriction enzyme. The results of the RFLP analysis can be performed by using different DNA electrophoresis technique. RFLP analysis is the cheapest and most widely used methods for genome mapping, genetic disorder, paternity and interspecies as well as interspecies variation analysis. However, recently, it got a wider acceptance in animal species authentication.

2.13.5.2 Design of primers and identification RFLP enzymes

The primers can be designed for PCR-RFLP assay by certain primer desiging program such as primer3 (Rozen & Skaletsky, 1999) in association with in-silico analysis to determine the suitable restriction enzyme for the specific fragment generated by the designed primers. In-silico analysis of the specific fragment to determine the restriction enzyme applicable for genetic or sequence variation can be done by the program different programs such as NEBcutter V2. This program is facilitated with in-silico digest option to view the yielding fragments (Vincze, Posfai, & Roberts, 2003). Recently, programs have been developed by integrating the selection of the primers along with restriction enzyme. Designing the PCR-RFLP assays using these programs, which is efficient in primer designing with appropriate restriction site and enzyme, may save significant amounts of time.

2.13.5.3 Electrophoretic separation and visualization of fragments

The PCR-RFLP assay is based on specific amplification of DNA fragment(s) followed by the restriction digestion of the PCR product using selected enzyme(s). The final end point detection of the resultant fragments is analyzed by electrophoresis. The most frequent electrophoresis method used for this is the slab gel electrophoresis with agarose or polyacrylamide as molecular sieving matrix. Recently new capillary electrophoresis and micro-channel electrophoresis have come forward with higher resolving power than conventional slab gels (Stellwagen & Stellwagen, 2009). Thus it is becoming a widely acceptable laboratory tool with higher resolving power and throughput.

The end point analysis of the PCR-RFLP assay can be done by visualization of restriction digested PCR product using labeled or unlabelled primers. In most of the assays with unlabelled primer, ethidium bromide or other dye is incorporated with

restriction digested products for visualization of the DNA fragments. For determining the exact product sizes of the restriction-digestion, the resulted fragments are pre heated to denature the double stranded DNA in to a single-stranded state. This procedure is best fitted for genotyping using the microsatellites markers. However, PCR-RFLP analysis with covalently labeled primer may be applicable for a variety of purposes.

2.13.5.4 Microchip electrophoresis in PCR-RFLP

The recent technological development allows the gel electrophoresis miniaturization and conduction in microchips. Compared to other electrophoretic DNA separation systems, the micro-fluidic based electrophoresis provides significant lower separation time and higher throughput (Sinville & Soper, 2007). The PCR-RFLP assay can be integrated with the microchip format, and can be analyzed by using several commercially available microfluidic DNA electrophoretic systems. Thus, several reports have been made on the application of PCR-RFLP assay for the species authentication based on microchip electrophoresis. It has been recommended for the application in quality control or regulatory screening in enforcement laboratories for screening or to detect undeclared admixture of fish species in seafood products (Dooley, Sage, Clarke, Brown, & Garrett, 2005). The Lab-on-a-chip based assays using capillary electrophoresis also have been documented for the authentication of coffee beans (Spaniolas, May, Bennett, & Tucker, 2006) and to determine pork in halal meat products (Ali, Hashim, et al., 2012).

2.13.5.5 Usefulness and limitation of PCR-RFLP Assay

PCR-RFLP is intrinsically more accurate methods than the species-specific PCR assay and can discriminate closely related species with authentication of the real PCR products (Doosti, Ghasemi Dehkordi, & Rahimi, 2011). However, PCR-RFLP is more

laborious and costly method than Species-specific PCR. PCR-RFLP assay with longer amplicon length may not useful for meat or meat product analysis due the breakdown possibility of the longer DNA templates which are very prone to degradation with thermal or food processing steps (Fajardo et al., 2010; Turna, 2010).

2.14 Application of PCR, PCR-RFLP and RT-PCR Assays in Species Detection

For the quality assurance of the food product and to protect consumer from purchasing adulterated or falsely labeled foods certain application of major conventional PCR based assays such as species specific PCR, PCR-RFLP and real time PCR assays for species detection have been described here.

2.14.1 PCR

a. Plant species detection in food

Application of PCR is one of the easy and user-friendly methods for the analysis of different plant originated food such as cereals (Terzi et al., 2005), legumes (Weder, 2002), allergens and with accent to nuts (Poms, Klein, & Anklam, 2004). The application of PCR assay is further extended to the identification of the plant variety and cultivar. It is also applied in the food analysis for the detection of additives, such as the spices (Remya, Syamkumar, & Sasikumar, 2004) and thickeners agents like locust bean gum (Meyer, Rosa, Hischenhuber, & Meyer, 2001). Nowadays, PCR may be the most widely applied methods for the routine analysis of genetically modified organism (GMO) detection in food, specially derived from soybean and maize.

The European Union (EU) legislation has protected certain important species of olive tree drupes (Oleaeuropaea L.) to obtain the olive oil typicity form specific conjunction with the climate conditions of the region and origin of the cultivar(s) (Mafra, Ferreira, & Oliveira, 2008). Thus the DNA based species identification method

was successfully applied in foods product analysis like which is prone to degradation and rich with PCR inhibitors (Breton, Claux, Metton, Skorski, & Bervillé, 2004). Certain cereals such as rye, barley and wheat contain storage protein (gluten) which in harmful patients with celiac disease leading the destruction of the small-intestinal mucosa (Poms et al., 2004). Hence, legislation has been made to ascertain the level of gluten by the restriction of different cereals used in the food product (Mafra et al., 2008). In association of the protein assay, Köppel et al., (1998) described a highly sensitive species-specific PCR (SSP) assay for the detection the cearals used in the foods (Köppel, Stadler, Lüthy, & HuÈbner, 1998). Furthermore, application of SSR analysis using species-specific PCR assay has enabled the detection of soft wheat in semolina and breads with a limit of 3 and 5% (w/w), respectively (Pasqualone et al., 2007). DNA based biomarker assay provide an essential tool for the detection of allergen-containing foods. For example, for analysis of food ingredients to determine the presence of hazelnut allergen, species-specific PCR assay allow the detection of 0.001% (w/w) of hazelnut in commercial food products using Cor a 1.0401 gene (Holzhauser, Wangorsch, & Vieths, 2000).

b. Fish species detection

There are certain PCR based assays for the detection of fish species. For analysis of processed food most of those assays are based on of mitochondrial DNA target. Documentation have been made for the detection of fish species (sole, Greenland halibut, megrim, Xounder, turbot) in Raw and frozen state species-specific PCR based on 5SrDNA (Céspedes et al., 1999).

c. Animal species detection

For earning more profit, most frequent form food adulteration in meat product is the addition of pork (*Sus scrofa*) to beef (*Bos taurus*). The low cost meat species such as pork or dog is not acceptable in the food due to certain reason, for example there are food taboos from certain religious view and dietary and health thread such as allergies or the introduction of food-borne pathogenic microbiological infection. Thus species specific PCR was used for the detection of porcine DNA from pork along with other species (Rodríguez et al., 2004). Furthermore, species-specific PCR techniques were also widely used for detection of meat species such as goat (Kumar, Singh, Singh, & Karabasanavar, 2011), sheep (Karabasanavar et al., 2011), beef (Mane, Mendiratta, & Tiwari, 2012), buffalo (Mane, Mendiratta, Tiwari, & Bhilegaokar, 2012), chicken (Mane, Mendiratta, & Tiwari, 2009; Stamoulis, Stamatis, Sarafidou, & Mamuris, 2010) from raw and admixed meat products.

2.14.2 PCR-RFLP

The application PCR-RFLP assay in different species authentication of food or food products are described below

a. Fish and Seafood

Recently, the consumption of the sea foods and derived products has been increased due to the awareness of the consumer on certain health and nutritional factors (Mafra et al., 2008). The taxonomic classification methods based on morphological characteristics, such as the size, shape, skin pigmentation and appearance usually can be applied to determine some of the world's fish catch those are sold unprocessed. Thus it enables the easy identification of species through rigorous inspection. However, certain commercially available products after removal of some external features (eviscerated, beheaded, skinned, etc.) and processing (marinated, salted, smoked, canned, frozen,

etc.), it is difficult to identify the species of origin of the foods. Thus, the EU labeling regulations specify that the commercial and scientific names should be included on the label of seafood products.

Table 2.2 PCR-RFLP based assay for the authentication species in seafood products

Species	Target	Product	References
Salmonids	Cytb	Raw and processed (smoked, heat-treated, marinated, canned, pastry)	Dooley et al., 2005; Hold et al., 2001
Flat Fish (sole, Greenland halibut, megrim, Xounder, turbot)	12S rRNA	Raw and frozen	Céspedes et al., 1999; Comesaña, Abella, & Sanjuan, 2003
Serranidae (Nile perch, grouper, wreck fish)	Actin	Raw and heat-treated	Asensio et al., 2001
Eels	Cytb	Raw and hot-smoked, canned	Rehbein et al., 2002
Gadoids	Cyt b 16SrRNA Mitochondrial control region	Raw, frozen, salted and heat-treated	Calo-Mata et al., 2003; Comi, Iacumin, Rantsiou, Cantoni, & Cocolin, 2005; Di Finizio, Guerriero, Russo, & Ciarcia, 2007; Quinteiro et al., 2001
Tuna	Cytb b	Raw, frozen and canned	Quinteiro et al., 1998
Sardines	Cytb	Raw and canned	Jérôme, Lemaire, Bautista, Fleurence, & Etienne, 2003
Sturgeon	Cytb	Caviar	Wolf, Hübner, & Lüthy, 1999
Clams	Actin	Raw and frozen	Fernández et al., 2000
Mussels	Adhesive protein/18SrDNA/ITS1	Raw	Santaclara et al., 2006
Cephalopods	FINS,Cytb	Raw, rings and canned	Chapela, Sotelo, & Pérez-Martín, 2003

Hence it is important to apply molecular species authentication techniques to determine the species in processed seafood. DNA-based methods facilitate over the protein-based methods it was widely used for the analysis of seafood products (Mackie et al., 1999). Thus the applications of PCR-RFLP assay to determine the species in a wide range of fish and seafood products are resumed in Table 2.2.

b. Meats and Dairy Products

PCR-RFLP assay is one of the widely used methods for determining the fraudulent labelling of the meat products usually composed of one or more species (Pascoal, Prado, Castro, Cepeda, & Barros-Velázquez, 2004). For example, it was successfully applied to explore the presence of commercially valuable turkey species in the meat product or the absence of the labelled expensive meat such as beef or roe-deer. Wolf et al. (1999) describes the application of the PCR-RFLP technique using two restriction digestion enzymes to discriminate 25 game species (Wolf, Rentsch, & Hübner, 1999). Thus this assay technique was helpful to determine the meat frauds associated with the alteration of expensive game meat and derived products.

Maede (2006) has described a strategy for molecular species detection in meat and meat products using mitochondrial and chromosomal genetic sequences by combining the PCR-RFLP assay with DNA sequencing. R. Meyer, Höfelein, Lüthy, and Candrian (1994) had presented the successful amplification of mitochondrial cytochrome b gene and analysis of endonuclease based digestion pattern for the detection of beef, pork, sheep, goat, buffalo, horse, turkey and chicken from processed, fermented or marinated products. Furthermore, Fajardo et al., (2006) documented the application of PCR-RFLP assay for the determination of meats from goat (Capra hircus), cattle (Bos Taurus), sheep (Ovis aries), red deer (Cervus elaphus), roe deer (Capreolus capreolus), and fallow deer (Dama dama).

Determination of the origin of species for the dairy products is an essential concern to safeguard the consumer health and religious faith. Thus, it will protect the consumer rights from economic loss as well as from the food borne allergens. The common form of dairy product adulteration includes the substitution of higher value milk by non-labeled cow's milk or the omission of a labeled milk species. Thus, the detection of milk species become an important issue in cheese making due the manufacturing of cheese from pure with protected designation of origin (PDO) such as pure sheep or pure goat's cheeses or milk from certain amount of milk from multiple species (Bottero, Civera, Anastasio, Turi, & Rosati, 2002). In line with these, Plath et al., (1997) have documented a PCR-RFLP assay for detection of goat, sheep, cow and buffalo casein genes (Plath, Krause, & Einspanier, 1997). The assay was proven to have high sensitivity with a detection limit of 0.5% (w/w) of cow's milk in goat's or ewe's milk cheeses. Lanzilao et al. (2005) describes a PCR-RFLP assay targeting the cytochrome b gene for the detection of dairy products from cow, sheep, goat and buffalo (Lanzilao, Burgalassi, Fancelli, Settimelli, & Fani, 2005). In another experiments, the PCR-RFLP assay allowed the detection of 1% (w/w) of cow's in buffalo milk cheeses (Bottero et al., 2002).

2.14.3 RT-PCR

The real time PCR allows us to determine the DNA target in samples by measuring the accumulation of amplified products using fluorescent technology as described above. Some of the applications of this widely applied method to fulfill the need to quantify the species in food materials are listed in Table 2.3.

Table 2.3 Application of Real time PCR for species detection from different food matrices.

Food product	Species	Target gene	Detection limit	References
Mozzarella cheese	Cow, buffalo	Cytochrome-b/growth hormone	0.1%	Lopparelli, Cardazzo, Balzan, Giaccone, & Novelli, 2007
Raw and heat treated milks	Goat, sheep	12SrRNA	0.5%	López-Calleja et al., 2007
Olive oil	Olea europeaecultivars	microsatellite DNA	NR	Breton et al., 2004
Gluten in food	Wheat, barley, rye, oats	ω-gliadin (Wheat) ω-secalin (rye), hordey (barley), avenin (oat)	DNA/0.01–0.1%	Sandberg, Lundberg, Ferm, & Yman, 2003
Food allergens	Hazelnut	hsp1	DNA/0.01%	Piknová, Pangallo, & Kuchta, 2008
Food allergens	Peanut	Ara h 2	< 0.001%	Hird, Lloyd, Goodier, Brown, & Reece, 200
Food allergens	Mustard	2S albumin	0.005%	Mustorp, Engdahl-Axelsson, Svensson, & Holck, 2008
Food allergens	Sesame	Sin A	0.5 pg DNA/0.005%	Mustorp et al., 2008
Meatball	Pork	Cytb	0.01%	Ali et al., 2012
Burger	Pork	Cytb	0.01%	Ali, Hashim et al., 2012
Nugget	Pork	Cytb	0.01%	Ali, Hashim, Shuaimi et al., 2012

NR not reported

2.15 Prospect of Proposed Canine DNA Biomarker Based PCR Assays

For animal species detection in food, the major biomarker based assays were based on analysis of lipid (Rohman et al., 2011), proteins (Asensio et al., 2008), and DNA were proposed (Ali, Hashim, et al., 2012) (Chapter 2, Section 2.12). However, the lipid based methods have the drawbacks in species authentication of process food due to the alteration of the positional distribution of the fatty acids during cooking (Chapter 2, Section 2.12.1.3.). For food adulteration detection from complex matrices, the specificity of the assay of the specific target based on VOCs analysis still need to well define (Chapter 2, Section 2.12.2.3). The protein based biomarkers assays cannot be utilized in for thermally treated food products due to the denaturation of soluble protein. Furthermore certain protein based assay has limitation on the specificity of the assay with the false positive result with close related species (Chapter 2, Section 2.12.3.3). However, DNA based PCR assays are widely used technique for species detection from different matrices (Chapter 2, Section 2.13). Therefore, the proposed DNA biomarker based canine species detection assay will provide opportunity to overcome these limitations to detect the canine species either from cooked or thermally treated processed food products. DNA based biomarkers assay provide a high discriminating power to distinguish closely related species (Ali, Hashim, et al., 2012). Among the DNA based methods different PCR assays are widely used methods for species authentication from different raw or processed foods (Chapter 2, Section 2.14). Thus for canine species detection five different conventional species specific PCR based assays using agarose gel electrophoresis technique have been proposed (Abdel-Rahman, El-Saadani, Ashry, & Haggag, 2009; Abdulmawjood, Schönenbrücher, & Bülte, 2003; Gao, Xu, Liang, Zhang, & Zhu, 2004; İlhak & Arslan, 2007; Martín et al., 2007). However, most of these assays have longer DNA target (>150 bp) which have the possibility of false negative result (Ali, Hashim, et al., 2012). Several reports have been

made for the breakdown of longer DNA target under extensive food processing conditions leading to the failure of the PCR assay (Arslan et al., 2006; Martinez & Malmheden Yman, 1998; Matsunaga et al., 1999). Only Martin et al., (2007) have reported a PCR assay targeting 101 bp fragment of canine 12S rRNA gene. However the assay was not validated under commercial food products background, while most of the food adulteration reports have been made in the minced meat or process food products. Short length DNA biomarkers based assay may amplify more efficiently and can be separated with higher resolution with a better recovery rate (Ali, Hashim, et al., 2012). Thus, the proposed short amplicon based DNA biomarker assays will eliminate the probability of the breakdown of the target with detection of canine species with higher accuracy and can be applied for the processed food analysis. It has also the potentiality for its application in the development biosensor (Jung, Mun, Li, & Park, 2009) and biochip to recover the DNA target from highly degraded food samples (Aboud, Gassmann, & McCord, 2010). Reports have been made for the utilization of the multicopy mitochondrial gene target for increase efficiency in forensic or highly degraded samples analysis (Butler, 2006). Mitochondrial cytochrome b specific assay have been reported for a higher specificity and sensitivity (Ali, Hashim, et al., 2012). Hence, the proposed canine specific assays targeting mt-cytb gene will allow more available DNA template for effective canine species detection with a higher sensitivity. Furthermore digestion of the proposed short-length target DNA with restriction endonuclease enzyme will increase the authenticity of the assay (Chapter 2, Section 2.13.5). The proposed canine DNA biomarker with widely used PCR-RFLP assay with micro-fluidic technology using lab-on-a-chip provides will provide more effective way for PCR endpoint detection (Chapter 2, Section 2.13.5.4). It will provide a canine species detection platform with higher resolution and speed and better reproducibility than the conventional agarose gel electrophoresis assay. Real-Time-PCR or qPCR assay

is a well described method for quantitative species authentication with higher sensitivity (Chapter 2, Section 2.12.4.3; 2.14.3). However, all previously documented PCR assays for canine species detection were based on qualitative analysis and none were tested in any commercial product. Therefore, the proposed research will also develop a quantitative assay platform with its potential application in process meat product such as nuggets.

CHAPTER 3
CANINE SPECIFIC PCR ASSAY TARGETING CYTOCHROME B GENE FOR THE DETECTION OF DOG MEAT ADULTERATION IN COMMERCIAL FRANKFURTERS

(Food Analytical Methods 7(1), 234-241)

3.1 Introduction

3.1.1 Frankfurter

Frankfurter is an emulsion type cooked sausages. It originated from Germany, and was named according to the German state, Frankfurt (Srinivassane, 2011). Nowadays, it has achieved popularity and appeal all over world. In United States, the annual sale of different branded frankfurters was $2024.4 million in 2012 (Figure 3.1) (Information Resources, 2013). In Malaysia, it is a highly consumed meat product and has gained popularity from school children to grown up individuals (Huda, Wei, Jean, & Ismail, 2010; Özvural & Vural, 2008). The manufacturing ingredients of frankfurters may vary from country to country, depending on the consumer choices and availability of the meats which is its major constituent. However, the name of the frankfurter as dog or bow-wows was not a pleasant one. The sausages producers in the nineteenth century were generally believed, not beyond the reason use of dog and horse meat in sausage preparation (Rawson, 2011). Furthermore, the recent meat scandals made it essential to develop methods for detection of canine meat from the process foods such as the frankfurter sausages.

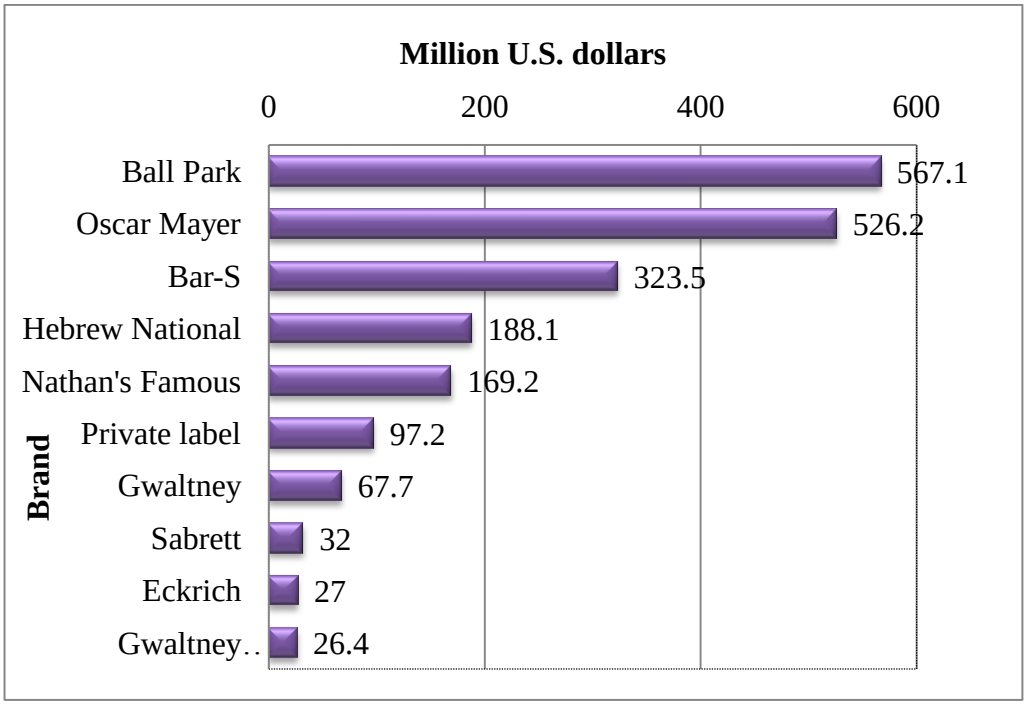

Figure 3.1 Sales of the leading 10 refrigerated frankfurter brands of the United States in 2012 (in million U.S. dollars) (Information Resources, 2013).

3.1.2 Current meat scandals in sausages

Date	Product	Event	References
2013-03-08	Sausages and ham	In Poland, rotten meat with green mould was found to sale in UK, Ireland, Germany and Lithuania after cleaning and drying to make sausages and ham. Furthermore, horse meat was labelled as beef in three Polish meat-processing plants.	Easton, 2013
2013-02-27	Frankfurter Sausages	Russian officials revealed horse DNA in the Frankfurter sausages from Austria.	Langlois, 2013
2013-02-27	Sausages	The Swedish giant company IKEA withdraws sausages from stores in France, Britain, Spain,	Ritter, 2013

		Ireland and Portuguese after the horse meat scandal for meatballs.	
2013-02-25	Beef burger and sausages	In South Africa, meat of water buffalo, goat and donkey was found in various meat products such as beef burger and sausages	Janice & Jaco, 2013
2013-02-21	Sausage	German supermarket chain 'Coop' had withdrawn around 20 horsemeat sausage products.	France, 2013
2012-11-30	Salami	The qualitative analysis revealed from a total of 19 beef salami products 10 were positive for bovine (beef) DNA but all were positive for porcine (pig) DNA.	Reilly, 2013

3.1.3 Prospect of Current Canine Species Detection in Frankfurter

Dog meat has been used for human consumption for decades in many parts of the world including South Korea, China and Vietnam (Podberscek, 2009). Since stray dogs are available in many countries of the world without any offered price (Kumarapeli & Awerbuch-Friedlander, 2009; Totton et al., 2010), fraudulent mixing of dog meats with costly halal meats would offer additional profit. In Malaysia, reports have been made of dog meat being eaten by foreign workers, especially from Myanmar (Nagpal, 2008). No systematic studies have been conducted for the detection of dog meats although there is a huge sale of frankfurter (Figure 3.1). To detect dog meats or its derivatives, several PCR based assays have been proposed (Abdel-Rahman, El-Saadani, Ashry, & Haggag, 2009; Abdulmawjood, Schönenbrücher, & Bülte, 2003; Gao, Xu, Liang, Zhang, & Zhu, 2004; İlhak & Arslan, 2007; Martín et al., 2007). However, most of them are having longer amplicon targets and may not be stable under extensively processed conditions (Ali, Hashim, Mustafa, & CheMan, 2012; Rojas et al., 2010). Furthermore, these assays were not tested under any commercial food matrices such as frankfurter formulation.

For the analysis of canine meat detection in highly processed foods, shorter DNA fragment (<150) based PCR assays are highly desirable. Under complex DNA pool which are often found in commercial food products, short amplicon length PCR assays aid by giving single PCR product rather than multiple bands which may arise from DNA fragmentation (Ali et al., 2012). Therefore, the objective of this work was to develop a shorter DNA fragment based PCR assay suitable for the detection of dog meats in commercial frankfurter which is a popular food item all over the world including Malaysia with a high economic value.

3.2 Materials and Methods

3.2.1 Collection of samples

Meat samples of eight commonly used meat species, namely chicken, duck, turkey, goat, sheep, beef, buffalo and pig, the most consumed four fish species, namely tilapia, rohu, tuna, and shrimp, and the five most common plant materials including rice flour, wheat flour, maize flour, garlic and nut-meg were purchased from the various supermarkets in Kuala Lumpur and Selangor in Malaysia. The dog meat was collected from the Faculty of Veterinary Science, University Putra Malaysia and Dewan Bandaraya Kuala Lumpur, Malaysia. All meat samples were transported under ice chilled condition (4 ^{0}C) and were stored at -20 ^{0} C for future work and DNA extraction.

3.2.2 DNA extraction

DNA was extracted from 25 mg of raw and processed meat samples using NucleoSpin® Tissue extraction kit (Macherey-Nagel, Germany) following the manufacturer's instructions. DNA from plant sources such as rice flour, wheat flour, maize flour, garlic and nut-meg powder was extracted using Cetyl Trimethyl Ammonium Bromide (CTAB) buffer as described in the figure 3.2. CTAB methods was applied with subsequent purification steps to get good quality DNA from admixed, dummy or commercial samples with higher sample size (100mg-1gm). Utilization of the commercial kit for the extraction of DNA from pure samples either raw or treated (25mg) was yielding applicable DNA for PCR assay. However, to obtain a good quality and sufficient amount of DNA from the admixed, model or commercial food matrices, it was necessary to increase the sample size (≥100mg) with subsequent purification steps. Since CTAB reagents are relatively cheap, it allowed us to extract DNA from increased sample volume, minimizing cost. Ready to eat or commercial meat products are composed of complex DNA matrices with additional feed additives. Furthermore, DNA

extraction using conventional methods may associated with contaminants and enzyme inhibitors. Therefore, we have used additional phenol free purification kits and obtained a good quality DNA for PCR assay. Qualitative DNA analysis was done by visualizing the bands after running the total DNA in 1 % agarose gel (Promega, Madison, USA) in 1x LB buffer of pH 8.0 at a constant voltage of 170 V for 20 min. Concentration and purity of the DNA was analysed by Eppendorf UV-vis Biophotometer (Eppendorf, Germany).

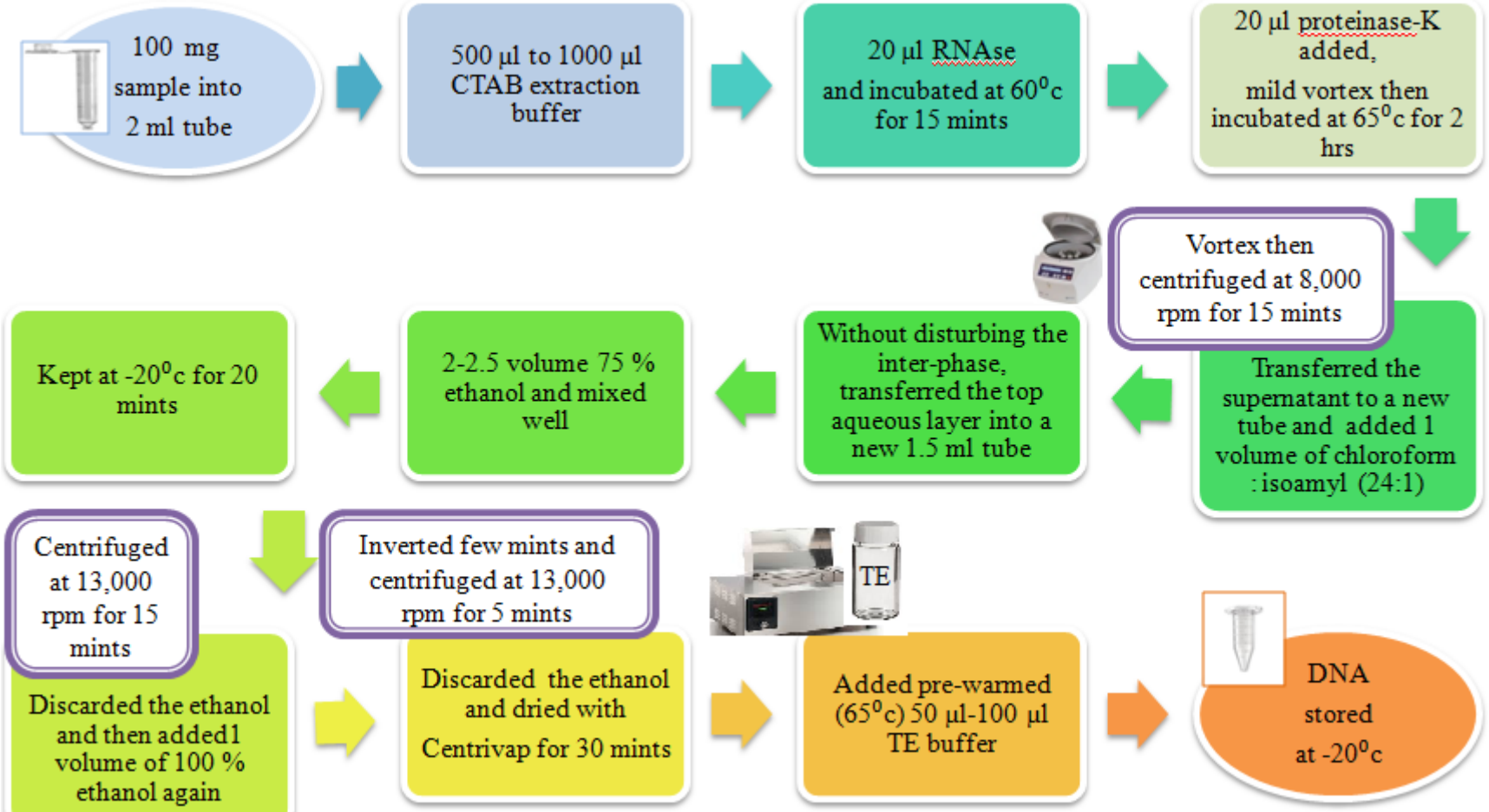

Figure 3.2 DNA extraction protocol using CTAB method

3.2.3 Canine specific biomarker

For canine specific biomarker, a pair of primers (Forward: 5′ CCTTACTAGGAGTATGCTTG 3′ and Reverse: 5′ TGGGTGACTG ATGAAAAAG 3′) defining a 100bp region of dog cytb gene was developed using a publicly available primer3plus Software (www.bioinformatics.nl/cgi-bin/primer3plus/primer3 plus.cgi) following (Ali, Hashim, Mustafa, & Che Man, 2012). The primers were screened for unique canine specificity to eliminate potential non-specific primer binding to the DNAs of other animal or plant species using online "BLAST" local alignment tool in NCBI data base (http://www.ncbi.nlm.nih.gov/blast). The primers were purchased from the 1st BASE Laboratories, Pte. Ltd., Selangor, Malaysia.

3.2.4 PCR assay optimization

The polymerase chain reaction was performed in a 20 µl of total reaction volume composed of 1x PCR master mix (Promega Corporation, Madison, USA) with 50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer of pH 8 containing 400µM each of dATP, dGTP, dCTP, dTTP and 3mM $MgCl_2$, 100 nM of each primer and 20 ng of total DNA. PCR was performed in a gradient thermocycler (Eppendorf, Germany) using an initial denaturation at 94^0C for 3 min followed by 30 cycles of denaturation at 94^0C for 30 s, annealing at 58^0C for 30 s and extension at 72^0C for 1 min. The final extension was performed at 72^0C for 5 min. The PCR products were stored at -20^0C for further analysis. The separation of PCR products, pre-stained with 6x loading dye, was performed in 1 % agarose gel (Promega, Madison, USA) in 1x LB buffer of pH 8.0 at a constant voltage of 170 V for 20 min using a 100 bp DNA ladder (Fermentas, USA). DNA visualization was performed with ethidium bromide staining under a gel documentation system (AlphaImager HP; Alpha Innotech Corp., San Leandro, California, USA).

3.2.5 Species specificity

To assess the canine specificity of the PCR assay, three methods were followed. Firstly, theoretical specificity was analyzed using bioinformatic softwares. The retrieved cytb gene sequences of animal and cob gene sequence of plant were aligned with the primer sequences using BioEdit version 7.0 and ClustalW sequence alignment tool (Thompson, Higgins, & Gibson, 1994). Secondly, experimental analysis was performed in a real PCR amplification reaction against 9 animal, 4 fish and 5 plant species. Finally, the purified PCR product was sequenced in triplicates and the sequences were matched through BLAST analysis in NCBI data base. The PCR product was purified using QIAquick PCR purification kit (Qiagen, USA) and the sequencing was done by the 1st Base Laboratories Pte. Ltd., Selangor, Malaysia. The sequences of the different animal, plant and fish species were used to construct a dendogram showing the genetic closeness and distance among the studied species using molecular evolutionary and phylogenetic analysis software, MEGA version 5.(Tamura et al., 2011).

3.2.6 Meat processing

The meat samples were cut into small pieces and were subjected to various processing treatments. The raw cut meat samples were boiled for 90 min at 100 ^{0}C to simulate traditional cooking. The pasteurization was performed according to European legislation (Commission, 2002) by heating the meats at 120 °C for 50 min, 110 °C for 120 min, and 133 °C at 45 psi for 20 min. Finally, extensive autoclaving which breaks down the target DNA was executed at 120 °C under 45 psi for 2.5 h (Ali et al., 2011). All the treated samples were stored at -20 ^{0}C.

3.2.7 Specificity under mixed background

A complex pool of heterogeneous DNA mixture was prepared by spiking variable amount of dog meat (0.01-10%) into 1:1 mixture of chicken and beef. Each specimen of the ternary admixtures contained a total of 100 g meats of dog, chicken and beef in the ratio of 10:45:45, 5:47.5:47.5, 1:49.5:49.5, 0.1:49.95:49.95 and 0.01:49.995:49.995 to realize 10%, 5%, 1%, 0.1%, and 0.01% of dog meat contaminated ternary meat mixtures. All admixtures were vigorously blended in replicates on three different days and were autoclaved at 120 °C under 45-psi pressure for 2.5 h.

3.2.8 Frankfurter preparation

Dummy chicken and beef frankfurters were prepared according to Savic et., al (1985). The negative controls were prepared using pure beef and chicken meats along with fats and seasoning (Table 3.1), and the positive controls were prepared by spiking into 10%, 5%, 1%, 0.1% and 0.01% of dog meat into the chicken and beef meats. The specified amount of chicken, beef and dog meats (Table 3.1) were thawed then cut into small stripes, grounded with a manual grinder of 3 mm plate (Hobart, USA) and mixed with one third of ice and seasoning (Table 3.1) for 5 min. Subsequently, the trimmed fat and the rest of ice were added and mixed well for 10 min. From this admixture, individual frankfurter was made by stuffing with commercial halal cellulose casings (Zxchem, China) and was hanged for 1 h to dry in room temperature. To get hot smoke effect, all frankfurters were kept in hot oven at 70 ºC for 60 min. The traditional cooking was simulated through cooking the frankfurter in hot water for 15 min at 75 ºC until the internal meat temperature was reached to 68-70 ºC. Thus prepared frankfurters were rinsed in cold water for 5 min and then stored at -20 ºC for subsequent DNA extraction.

Table 3.1 Ingredients level for 100g frankfurters using chicken and beef meat

Ingredients	Chicken frankfurter	Beef frankfurter
Chicken meat	80g	
Beef meat		50g
Chicken fat	10g	
Beef fat		35g
Wheat flour	2g	
Ice	8g	15g
Seasoning		
Nitrite salt	1.80g	2.30g
Dextrose	0.20g	0.03g
Black pepper	0.15g	
White pepper		0.23g
Red pepper	0.12g	
Coriander	0.05g	0.02g
Mace		0.03g
Monosodium glutamate	0.02	
Thyme	0.05	
Phosphate	0.04	
Sodium nitrite		0.04g
Chili		0.01g
Sage		0.01g
Nut-meg		0.04g
Garlic		0.03g
Sugar		0.3g
Sodium ascorbate		0.04g

0.01%, 0.1%, 1%, 5% and 10 % of dog meat was admixed with these formulations for canine meat positive frankfurters preparation

For commercial food product analysis, chicken and beef frankfurters from 3 different brands (labelled as A-C) were purchased in triplicates from Malaysian supermarkets located in Selangor, Petaling Jaya, and Kuala Lumpur in Malaysia. All dummy and dog meat contaminated frankfurters were prepared in 3 replicates on 3 different days.

3.3 Results and Discussion

3.3.1 DNA extraction and PCR amplification

Extractions of DNA from raw and thermally treated meat samples were performed using a commercial kit (Macherey-Nagel, Germany) that is known to reduce the loss of DNA during aqueous and organic phase separation and give a high yield of DNA (Karabasanavar et al., 2011). The highest DNA yield was obtained from the raw dog meat (180-230 ng/mg) and the lowest from the extensively autoclaved meat samples (90-102 ng/mg). This might be due to the degradation of genomic DNA to a certain extent under prolonged heat and pressure processing (Ali et al., 2011). A higher sample size (100 mg) was used to extract DNA from admixed meat and frankfurters using a modified CTAB method, popularly known to give higher yield of DNA. The yield of DNA was higher (430-456 ng/mg) in admixed samples than those of frankfurters (322-360 ng/mg). This might be due to the presence of plant materials and higher content of fat in frankfurter formulations (Table 3.1). The purity (A260/A280) of all DNA samples was 1.90–2.0 in triplicates. Primer annealing at a higher temperature increases specificity and eliminates nonspecific hybridization (Ali et al., 2012). Therefore, an optimized high annealing temperature (58 ^{0}C), as determined through repeated run of gradient PCR, was used for the amplification of the selected region of cytb gene from raw, treated, admix and commercial samples.

3.3.2 Canine specificity

To determine the canine specificity of the developed primers, cytb and cob gene sequences of dog and other 17 animal, fish and plant species were retrieved from NCBI database (Dog: JF489119.1, Chicken: EU839454.1, Beef: EU807948.1, Water buffalo: D32193, Domestic duck: HQ122601.1, Turkey HQ122602.1, Sheep: EU365990.1,

Goat: EU130780.1, Pig: GU135837.1, Tilapia: AF015020.1, Rohu: JQ346135.1, Tuna: AM989973.1, Shrimp: EU069446.1, Rice: X17064.1, Maize X00789.1, Wheat: X02352.1, Nutmeg: DQ916628.1, Garlic: AF356823.1). The canine specific primer pairs designed from the retrieved mt-cytb sequences showed perfect match with the canine cytb gene and more than 5 nucleotide variations with other species. In addition, the blast results showed 100 % identity with the canine species and eliminate the probability of primer binding with non-target species DNAs. In a real PCR run using DNAs from total 18 species including dog and other animals, fish and plant species, the assay successfully amplified only 100 bp of canine target (Figure 3.3 b). The sequencing result of the 100 bp PCR product also confirmed 100% similarity only with the canine (*Canis lupus familiaris*) cytb gene.

Species-specific PCR techniques are widely used for the detection of meat species from raw and admixed meat product such as sheep (Karabasanavar et al., 2011), beef (Mane, Mendiratta, Tiwari, & Bhilegaokar, 2012), pork (Che Man, Aida, Raha, & Son, 2007) etc. It has been implicated in several reports that the efficiency of the PCR assay might reduce or end up with failure in amplification in the presence of single mismatches in the primer binding region (Ali et al., 2012). The proposed mt-cytb based primer pair had multiple mismatches in the primer binding sites with the other tested species and in a real PCR run confirmed 100 % specificity only with the canine species. Previously, 4 different species specific PCR assays using mitochondrial whole genome (322 bp) (İlhak & Arslan, 2007), mitochondrial cytb (808 bp) (Abdel-Rahman et al., 2009; Abdulmawjood et al., 2003), D-loop (213 bp) (Gao et al., 2004) and 12S rRNA (101 bp) (Martín et al., 2007) have been proposed for dog meat detection. However, three of these assays used longer sized amplicon (≥213 bp) (Abdel-Rahman et al., 2009; Gao et al., 2004; İlhak & Arslan., 2007), which may not be stable under extreme physio-chemical and thermal conditions of food processing. The latest studies

appreciated short amplicon length PCR assay (<150bp) for the detection of species in highly processed foods (Ali et al., 2012). Martin et al., (2007) proposed a short-amplicon length PCR assay targeting 101 bp region of canine 12S rRNA gene which should be suitable for the analysis of processed foods. However, recent species identification schemes have showed that cytb-based assays are more accurate to reconstruct the mammalian phylogeny with higher resolution (Nicolas et al., 2012). Therefore, we expected that a mt-cytb based short amplicon length (100 bp) PCR assay would detect canine derived materials under raw as well in highly processed commercial foods.

Pair wise distances among the 18 studied species computed using the Maximum Composite Likelihood method (Tamura et al., 2011) were in the range of 0.26 to 2.29 (Table 3.2). The lowest distance was observed between dog and sheep and the highest was found between dog and garlic. To prevent non specific amplification of closely related species the mismatch bases in primer binding sites were 42 % to 55 %. The dendogram constructed by Neighbor-Joining method (Saitou & Nei, 1987) showed clear discrimination of the dog from other animals and plants species (Figure 3.3 a).

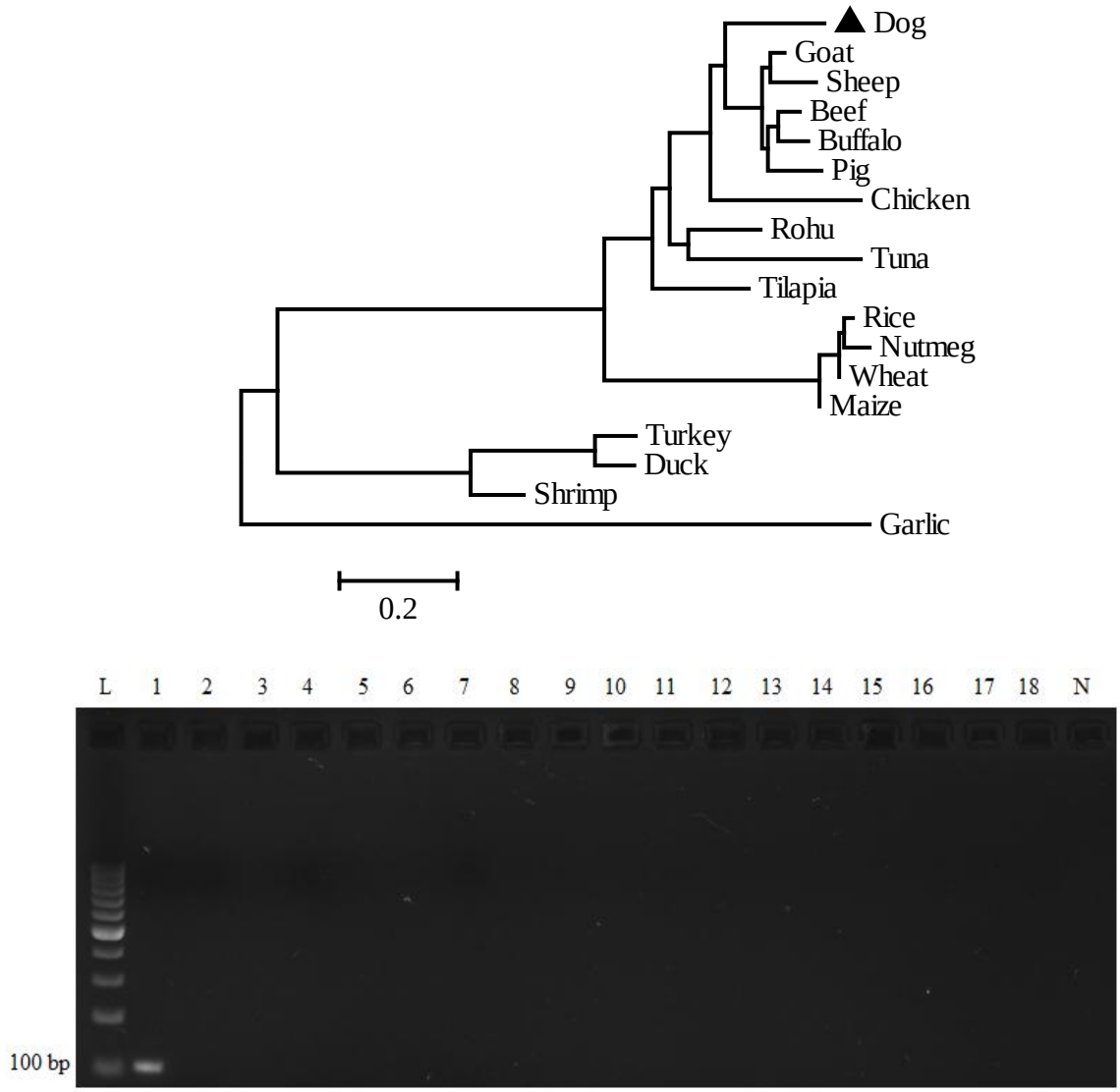

Figure. 3.3 Species specificity test of the mitochondrial cytb based canine specific PCR assay for frankfurter analysis. a) Dendogram showing clear discrimination of dog from 17 other species. b) Clear 100 bp product was obtained only from DNA extracted meats of dog but not from other common 8 animals, 4 fish or 5 plants species. Lane N: Negative control Lane L: 100 bp Ladder; Lane 1 to 9: DNA from dog, chicken, duck, turkey, sheep, goat, beef, buffalo and pig respectively; Lane 10 to 14: DNA from rice, maize, wheat, nutmeg and garlic respectively; Lane 15 to 18: tilapia, rohu, tuna, shrimp respectively.

Table 3.2 Pair wise distances between 100 bp cytb canine specific site and common meat, fish and plant species.

	Dog	Chicken	Turkey	Duck	Goat	Sheep	Beef	Buffalo	Pig	Rice	Maize	Wheat	Nutmeg	Garlic	Tilapia	Rohu	Tuna	Shrimp
Dog	0.00																	
Chicken	0.45																	
Turkey	1.20	2.00																
Duck	1.03	1.83	0.14															
Goat	0.27	0.42	1.22	1.20														
Sheep	0.26	0.52	1.25	1.28	0.10													
Beef	0.30	0.37	1.37	1.34	0.07	0.18												
Buffalo	0.35	0.37	1.46	1.48	0.14	0.15	0.09											
Pig	0.36	0.42	1.28	1.25	0.13	2.0	0.15	0.15										
Rice	0.75	0.82	1.77	1.77	0.79	0.68	0.83	0.77	0.72									
Maize	0.69	0.75	1.91	1.91	0.72	0.68	0.73	0.77	0.66	0.06								
Wheat	0.72	0.79	1.64	1.65	0.76	0.68	0.80	0.77	0.69	0.03	0.04							
Nutmeg	0.80	0.88	1.71	1.65	0.85	0.77	0.86	0.87	0.78	0.06	0.04	0.03						
Garlic	2.29	1.60	1.61	1.27	2.27	2.24	2.20	2.29	2.30	2.01	1.96	1.91	1.83					
Tilapia	0.51	0.52	1.49	1.69	0.39	0.51	0.35	0.41	0.43	0.68	0.55	0.65	0.65	1.83				
Rohu	0.42	0.47	1.14	1.57	0.36	0.41	0.38	0.38	0.38	0.83	0.76	0.08	0.90	1.92	0.31			
Tuna	0.61	0.69	2.01	2.00	0.57	0.51	0.57	0.53	0.55	0.83	0.73	0.08	0.86	1.84	0.48	0.41		
Shrimp	0.93	1.97	0.38	0.36	1.03	1.05	1.17	1.28	1.03	1.37	1.42	1.30	1.30	1.84	1.33	1.28	1.83	0.00

3.3.3 Target stability under thermal processing

The efficiency of the PCR assay under different thermal processing was tested and clear PCR products were obtained from all samples (Figure 3.4), reflecting the stability of this assay. Martín et al (2007) have proposed a PCR assay targeting a 101 bp region of 12S rRNA gene of canine species and tested it under various heat treatments in accordance with the European legislation (Commission, 2002). However, Martín et al (2007) did not test the stability and performance of their PCR assay under extensive autoclaving conditions which is known to breakdown target DNA (Ali et al., 2011).

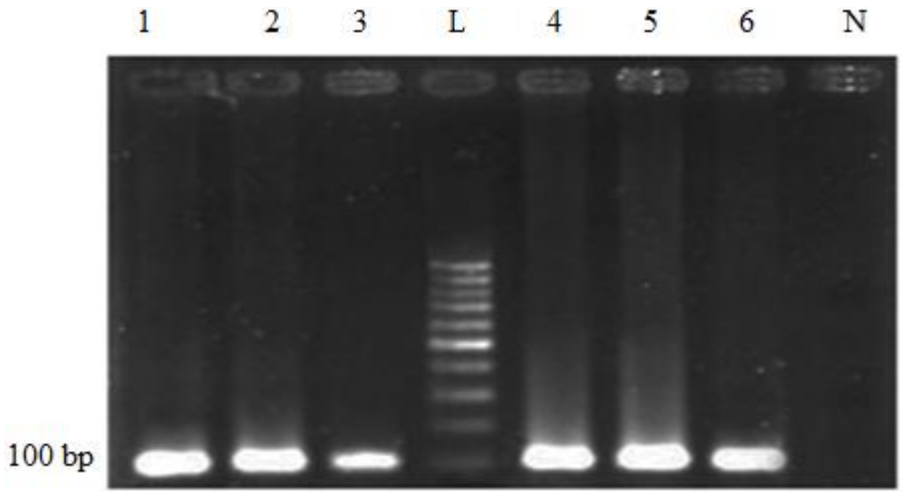

Figure 3.4 Stability analysis of the mitochondrial cytb based canine specific PCR assay under thermal processing conditions. Clear 100 bp PCR product was obtained from different thermally treated dog meat samples. Lane 1: Raw dog meat, Lane 2: 90 min boiled dog meat; Lane 3: Autoclaved dog meat at120 °C under 45 psi for 2.5 h.; Lane L: 100 bp ladder; Lane 4 to 7: autoclaved dog meat at120 °C for 50 min; 110 °C for 12 min, and 133 °C for 20 min under 43.51 psi respectively, Lane N: Negative control.

In the present assay, the 100 bp target of the canine cytb gene was obtained from the extensively autoclaved (2.5 h) dog meats, showing an extraordinary stability of the developed PCR assay. This was not surprising since (Ali et al., 2012) also documented an extraordinarily stable short length (109 bp) PCR assay targeting cytb gene for pork authentication in halal foods.

3.3.4 Performance at mixed meat background

Mt-cytb based canine specific PCR assay developed here successfully detected canine specific target from as low as 0.1% (0.02 ng DNA) of dog meat contaminated admixtures (Figure 3.5) under the excessive pool of chicken and beef DNAs, reflecting its high sensitivity and specificity even with complex background.

The sensitivity of the dog specific PCR assay documented by Martín et al., (2007) targeting a 101 bp fragment of 12S rRNA gene in meat-oats binary admixtures under normal autoclaving condition was 0.1% (0.125 ng). Compared to this assay, our assay was 6.25 times more sensitive in terms of the amount of template DNA used (125 ng vs. 20 ng) and in terms of mixed meat matrices (ternary meat mixtures) under extensive autoclaving. To the best of our knowledge, the highest detection limit (0.01%) for the detection of dog meat was reported by Abdulmawjood et al (2003) in a PCR-RFLP assay where the target was a 808 bp fragment of mitochondrial cytb gene. However, Abdulmawjood et al. (2003) did not describe the amount of DNA template they used in their analysis. A number of literatures reported that longer DNA-based PCR assays are less sensitive and less stable over the shorter ones under extensive food processing conditions (Ali at al., 2011). However, the percentage composition, which is an undefined method for concentration expression, can be manipulated through the use of higher amount of template DNA. Thus the sensitivity of the 808-bp length PCR assay cannot be higher than that of 101-bp PCR assay.

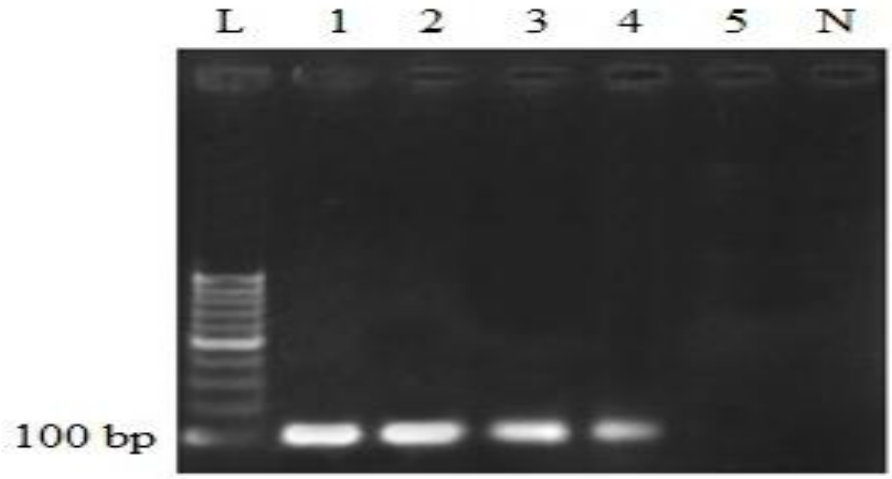

Figure 3.5 Performance analysis of the mitochondrial cytb based (100 bp) canine specific PCR assay at admixed background. Clear PCR products were noticed only from ternary admixtures composed of 10% to 0.1% of dog meat with chicken and beef. Lane L: 100 bp ladder; Lane: 1 to 5: 10%, 5%, 1%, 0.1%, and 0.01% of dog meat admixed ternary admixtures of chicken and beef respectively; Lane N: negative control.

3.3.5 Evaluation in frankfurters

Finally, the performance of the assay was tested in dummy chicken and beef frankfurters deliberately spiked with 0.01 to 10% dog meat. Clear PCR products were obtained from 0.1% to 10% dog meat spiked dummy chicken and beef frankfurters (Figure 3.6 a). Thus in a blind experiment, 0.1% dog meat spiked dummy frankfurters were used as a positive control for screening three halal (A-C) logo containing commercial chicken and beef frankfurters obtained from various super markets across Malaysia. No commercial samples were found to be positive for dog meat adulteration (Table 3.3 and Figure 3.6 b).

Table 3.3 Analysis results of frankfurters using canine mt-cytb (100 bp) based PCR

Frankfurter samples	Day 1	Day 2	Day 3	0.1 % dog meat positive samples	Detection probability
Dummy pure chicken frankfurter	3	3	3	0/9	100
Dummy pure beef frankfurter	3	3	3	0/9	100
Dummy dog spiked chicken frankfurter	3	3	3	9/9	100
Dummy dog spiked beef frankfurter	3	3	3	9/9	100
Commercial chicken frankfurter					100
A	3	3	3	0/9	
B	3	3	3	0/9	100
C	3	3	3	0/9	100
Commercial beef frankfurter					
A	3	3	3	0/9	100
B	3	3	3	0/9	100
C	3	3	3	0/9	100

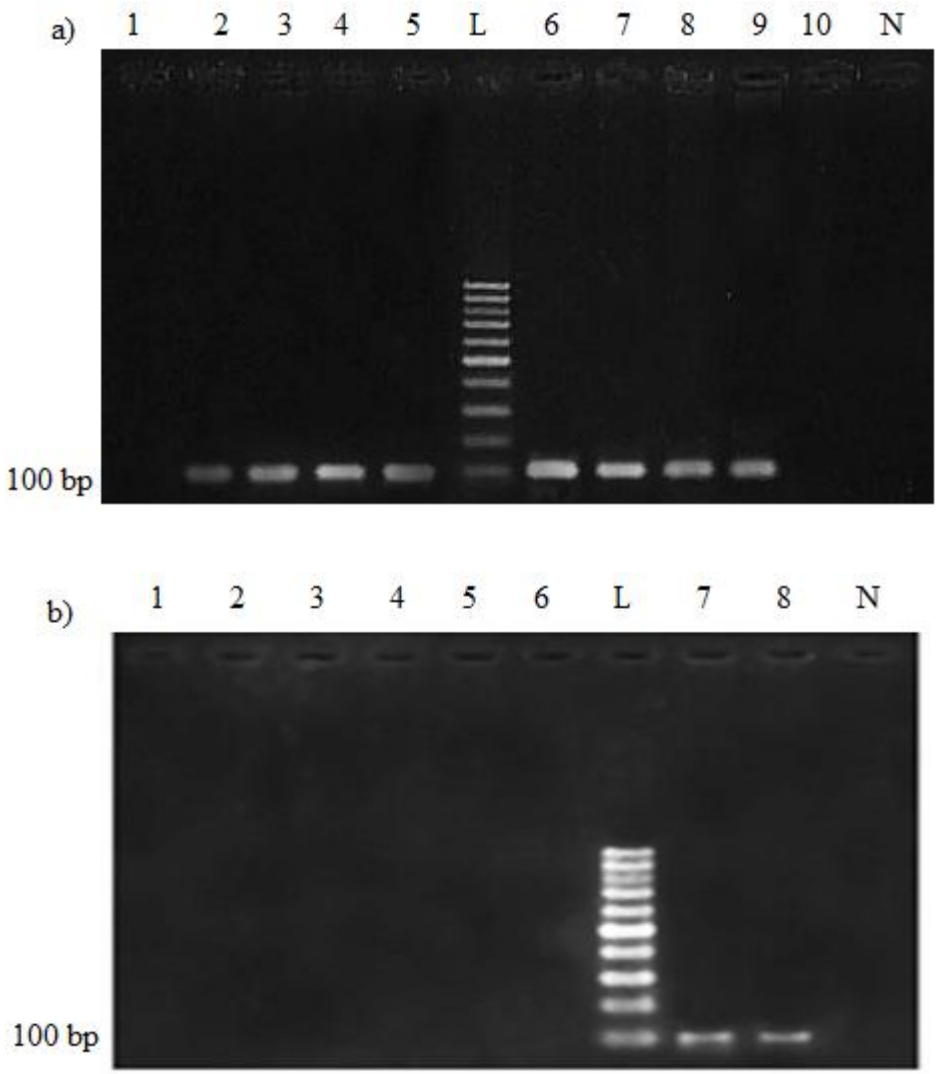

Figure 3.6 Frankfurter analysis using mitochondrial cytb based (100 bp) canine specific PCR assay. In a) 100 bp PCR products obtained from 10%, 5%, 1% and 0.1% of dog meat spiked dummy chicken frankfurters. Lane: 1-5: 0.01%, 0.1%, 1%, 5% and 10% dog meat spiked dummy beef frankfurters; Lane: 6-10: 10%, 5%, 1%, 0.1% and 0.01% dog meat spiked dummy chicken frankfurters respectively; Lane N: negative control. In b) No PCR product was obtained from commercial frankfurters except from 0.1% dog meat spiked chicken and beef dummy frankfurters. Lane 1-3: commercial chicken frankfurters; Lane 4-6: 1-3 commercial beef frankfurters purchased from Malaysian local market. Lane L: 100 bp Ladder; Lane 7 and 8: 0.1% spiked chicken and beef frankfurter respectively; Lane N: negative control

Although different PCR assays were previously proposed for canine meat detection (Abdel-Rahman et al., 2009; Abdulmawjood et al., 2003; Gao et al., 2004; İlhak & Arslan., 2007; Martín et al., 2007), none of them was tested for commercial meat products. But the presence of various additives and inhibitors in commercial meat and food products might prevent the primer binding at specific sites and reduce the amplification efficiency (Di Pinto, Forte, Conversano, & Tantillo, 2005). Therefore, we analyzed our assay performance with dummy chicken and beef frankfurter formulation. A constant detection limit of 0.1% (0.02 ng DNA) was obtained in all positive controls showing high performance under complex background of frankfurter, and may be due to the shorter size (100bp) of the cytb gene target, which is known for better sensitivity and stability under harsh conditions (Ali et al., 2012). Malaysian Government has a strong commitment to build a halal hub in local and international arenas (Talib, Ali, Anuar, & Jamaludin, 2008). Therefore, our study was in line with the government policy and we found the validity and applicability of our assay for the detection of canine tissues both in raw and processed commercial products.

3.4 Conclusion

A cytochrome b based short-amplicon length species specific PCR assay was developed and utilized to detect canine meat tissues in raw and processed meats as well as in commercial chicken and beef frankfurters. The assay has utilized a very short length (100 bp) target of the canine cytb gene and thus was very stable and sensitive under all potential food processing conditions. The specificity of the assay was tested against 9 meats providing animal species, 4 commonly consumed fish species and 5 common plant species. The detection limit of the assay was 0.1% of spiked dog meat in ternary meat mixtures and frankfurter formulations using 0.02 ng template DNA. We believe that this assay would find application in food industry for the authentication of canine origin materials in food products such as frankfurter.

CHAPTER 4
POLYMERASE CHAIN REACTION ASSAY TARGETING CYTOCHROME B GENE FOR THE DETECTION OF DOG MEAT ADULTERATION IN MEATBALL FORMULATION

(Meat science, 97(4), 404-409))

4.1 Introduction

4.1.1 Meatball

Meatball is a processed comminuted meat product. Its popularity is extended all over the world including Asia and Europe. Meatball is known as "bebola" in Malaysia and "bakso" in Indonesia. The most widely found meatball is prepared from beef meat, where as the halal meatball can also be prepared from chicken or lamb meat or fish (Purnomo & Rahardiyan, 2008). The Swedish furniture company IKEA alone had predicted to serve 700 million people around the world using it's 300 cafeterias with a quantity of 150 million meatballs in 2013 (Hansegard, 2013).

4.1.2 Current meat scandals in meatballs

Date	Product	Event	References
2014-05-16	Meatball	Mie Wonogiri meatball shop at Jakartha, Indonesia was proven to used wild boar in its meatball ingredient	Marbun, 2014
2013-02-27	Meatball	The Czech State Veterinary Administration had announced horsemeat had been found in meatball manufactured in Sweden and the Swedish furniture giant Ikea removed	Tomlinson, 2013

		its sausages from sale in Britain, France, Spain, Ireland and Portugal.	
2013-02-26	Meatball	The Swedish giant furniture company Ikea has drawn attention worldwide due to the Europe's widening food labelling scandal. The horse meat was detected in the frozen meatballs labelled as beef and pork in13 countries across the European continent.	Doward, 2013
2013-02-12	Meatball	Up market chain Waitrose became embroiled in a fresh row as its value range of frozen beef meatballs tested positive for pork	Sayid, 2013)
2012-08-30		Chicken meat that is spoiled for a few days or dead was used in the meatball instead of high price of beef meat.	Kristiawan, 2012
2011-03-05	Bakso/Indonesian meatball	In Indonesia, Television expose revealed that expensive beef/lamb meat is replaced by rat meat by some surveyor for the preparation of Indonesian bakso.	Allard, 2011
2010-04-28	Meatball soup	In "bakso" spicy meatball soup it was found to be made with Javan langur or silver-leaf monkeys found in Baluran National Park, East Java province, Indonesia	(Telegraph, 2010)
2007-08-11	Meatball soup	In Indonesia (South Sumatra) producer of bakso meatball soup was found to fill meatballs with pork instead of beef meat.	Patung, 2007

4.1.3 Prospect of Canine Species Detection in Meatball Formulation

As described above the Swedish furniture company IKEA alone had predicted to serve 700 million people worldwide with meatballs, but the giant company led a huge attention with the horsemeat scandal in meatballs (Hansegard, 2013). Using YouGov's brand perception tool, Brand Index, the analysis of the impact of this scandal in UK, it showed that Ikea's Buzz score had immediately decline (0.55) from a better stage (15) in comparison to other brands on 25 March, 2013 (Figure 4.1) (Dobrin, 2014).

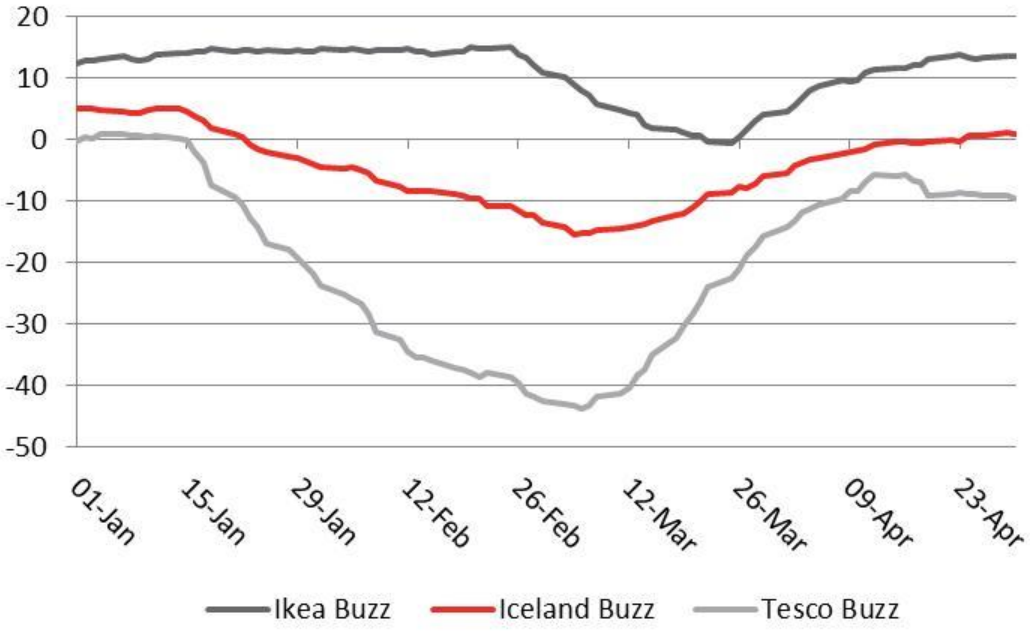

Figure 4.1 Buzz Score of different brands after the meatball scandal on IKEA (Dobrin, 2014).

For more profit, fraudulent admixing dog meat in meatball is a potential adulterant due to the availability of stray dog in many countries of the world without any price. However, for dog meat detection prior to our work, certain PCR assay using mitochondrial whole genome (İlhak & Arslan, 2007), mitochondrial cytb (Abdel-Rahman et al., 2009), D-loop (Gao, Xu, Liang, Zhang, & Zhu, 2004) and 12S rRNA (Martin et al., 2007) have been proposed. Most of these assays had larger amplicon size

(>150 bp) (Abdel-Rahman, El-Saadani, Ashry, & Haggag, 2009; Gao, Xu, Liang, Zhang, & Zhu, 2004; İlhak & Arslan, 2007). But latest studies demonstrated usefulness of short length PCR assay (<150bp) for species detection from highly processed food (Rojas et al., 2010). Furthermore, cytb-based PCR assay is more accurately reconstructed the mammalian phylogeny (Nicolas et al., 2012) and the possession of single allele, due to maternal inheritance, makes mtDNA unique as a powerful tool for tracking species ancestry, especially in its highly conserved cytb gene region (Brown, George, & Wilson, 1979; Murugaiah et al., 2009). Therefore, we developed a short amplicon based assay using cytb gene target and tested for its application in commercial food matrice like frankfurter (Chapter 3). Although there are lot of reports including the IKEA horsemeat scandal and meatball adulteration in Indonesia (Chapter 4, Section 4.1.2), there has been no work so far for detection of canine species in meatball formulation. Therefore, the present research was aimed to test and utilize the cytb based speceies specific PCR assay for the detetion of canine species in meatball formulation.

4.2 Materials and Methods

4.2.1 Samples collections

Meat samples of 9 commonly available animals (chicken, duck, turkey, quail, goat, sheep, beef, buffalo and pig) and 3 plants species (onion, garlic and tomato) were purchased in triplicates from various supermarkets located at Serdang, Petaling Jaya and Kuala Lumpur in Malaysia. The typical fresh dog and cat meats were collected from euthanized stray dogs and cats from Jabatan Kesihatan Dewan Bandaraya Kuala Lumpur (DBKL), Malaysia. The post-mortem dog meat samples were also collected from the Faculty of Veterinary Science in University Putra, Malaysia. Commercial meatballs of 5 different halal brands were purchased from Serdang, Petaling Jaya and Kuala Lumpur in Malaysia in triplicates on three different days. All the collected samples were transported under ice chilled condition (4^0C) and were stored at -20^0C for further processing and DNA extractions.

4.2.2 Meatball preparation

Pure meat balls were prepared according to Rohman, et al., (2011) with grinded chicken, beef and dog meat and balance ingredients added such as cooking salt, garlic, etc included in table 4.1. All the ingredients of different meat have been mixed and well blended. The emulsified meat mixtures were subsequently mechanically given ball shape. All the samples were stored at -20 °C for further processing and DNA extraction.

Table 4.1 Ingredients level for meatball preparation

Ingredient	Beef meatball	Chicken meatball	Dog meatball
Minced meat	100 gm	100 gm	100 gm
Breadcrumbs	7.5 gm	7.5 gm	7.5 gm
Chopped onion	5 gm	5 gm	5 gm
Ginger freshly chopped	1.5 gm		1.5 gm
Cumin powder	1.25 gm		1.25 gm
Garlic powder	1.25 gm	1.25 gm	1.25 gm
Black pepper		0.14 gm	0.14 gm
Milk		0.01 litre	0.01 litre
Butter		3.28 gm	3.28 gm
Tomato paste	2.5 gm		2.5 gm
Salt	0.05 gm	0.05 gm	0.05 gm

*With Beef and Chicken meat ball 0.01% to 1% of dog meat was added for contaminated sample preparation

4.2.3 DNA extraction

DNA was extracted from 25 mg of raw and treated meat samples using NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel, Germany) following manufacturer's instructions. For plant species, admixed and commercial samples, DNA extraction was performed from 100 mg specimen using appropriate CTAB method for

plant and animal DNA extraction as described in the chapter 3 sections 3.2.2. Subsequent purification was done by Promega Wizard™ DNA isolation kit (Promega Corporation, Madison, USA). Primary DNA analysis was done by gel image capturing after running the total DNA in 1% agarose gel containing 1µg/ml ethidium bromide in 0.5% Tris Borate buffer (TBE) for 45 min at 100 volt. The concentration and purity of DNA were determined using a spectrophotometer (Biochrom Libra S80 – Cambridge, England).

4.2.4 Canine specific Primer

The hyper variable region of the dog cytb gene (Dog: JF489119.1) was identified through alignment analysis with the cytb genes of 8 common halal meat species (Chicken: EU839454.1, Turkey HQ122602.1, Duck: HQ122601.1, Quail: EU839461.1 Beef: EU807948.1, Buffalo: D32193, Sheep: EU365990.1, Goat: EU130780.1), 2 non halal meat species (Pig: GU135837.1, Cat: AB194817.1) and apocytochrome b (cob) gene of 3 plant species (Tomato: XM004251454.1, Garlic: AF356823.1, Onion: GU253304.1) using mega 5 (Tamura et al., 2011) software and clustalW alignment tool (Thompson, Higgins, & Gibson, 1994). Thus found hyper variable regions were used to design a pair of canine specific primers (Forward 5′ CCTTACTAGG AGTATGCTTG 3′ and Reverse: 5′ TGGGTGACT GATGAAAAAG 3′) using primer3plus software (www.bioinformatics.nl/cgi-bin/primer3 plus/primer3plus.cgi). The canine specificity of the designed primers were theoretically confirmed through “BLAST” analysis in NCBI data base (http://www.ncbi. nlm. nih.gov/blast). Primers were purchased from the 1st BASE Laboratories Pte Ltd (Selangor, Malaysia).

4.2.5 PCR assay optimization

Canine Specific PCR assay was performed in a gradient thermocycler (Eppendorf, Germany), using 20 µl of reaction mixture composed of 1x PCR master mix (Promega, Promega Corporation, Madison, USA) containing 50 units/ml of Taq DNA polymerase (supplied in a proprietary reaction buffer pH 8, 400 µM each dATP, dGTP, dCTP, dTTP and 3 mM MgCl2), 100 nM of each primer and 20 ng of total DNA. PCR cycling was done using an initial denaturation at 94 ^{0}C for 3 min followed by 30 cycles of denaturation at 94 ^{0}C for 30 s, annealing at 58 ^{0}C for 30 s and extension at 72 ^{0}C for 1 min. The final extension was performed at 72 ^{0}C for 5 min. The separation of PCR products was performed in 1 % agarose gel (Promega, Madison, USA) in 1x LB buffer of pH 8.0 at a constant voltage of 170 V for 15-20 min, pre-stained with 6x loading dye and using a 100 bp DNA ladder (Fermentas, USA) as reference standard. PCR product was visualized on ethidium bromide stained agarose gel using a gel image documentation system (AlphaImager HP; California, USA).

4.2.6 Pair wise distance and phylogenetic tree

For pair wise distance and phylogenetic analysis, sequencing results obtained from 100 bp PCR product was aligned with the retrieved cytb/cob gene sequences of the tested species using ClustalW sequence alignment tool (Thompson et al., 1994). To study pair wise distance between dog and other aligned species, the consensus regions of the gene were used and a phylogenetic tree was built using molecular evolutionary and phylogenetic analysis software, MEGA version 5 (Tamura et al., 2011).

4.2.7 Canine specificity

The canine specificity of the assay was tested in three steps. In step 1, the retrieved DNA sequences were aligned along with the primer sequences using mega 5 (Tamura et al., 2011) software and clustalW alignment tool for mismatch detection. A 3D plot was built using the number of mismatches and pairwise distance data to find the unique discriminating properties of the primers and molecualr orbit using XLSTAT version 2013 (Addinsoft, 2013). In step 2, cross species testing was performed in a real PCR run using DNA extracted from the potential meat and plant species for meat ball preparation. Finally, PCR product was purified using QIAquick PCR purification kit (Qiagen, USA) and sequencing result were analyzed using BLAST local alignment tool in NCBI data base to confirm the canine cytb gene specificty. The sequencing was done by the 1st Base Laboratories Pte. Ltd., Selangor, Malaysia.

4.2.8 Specificity and sensitivity of the assay

To evaluate the performance of the assay under meatball matrices, various percentage of dog meat was added with chicken and beef meatballs formulations. To obtain dog meat contaminated meatballs 1%, 0.5%, 0.2%, 0.1%, and 0.01% of dog meat was added with 100 g of chicken and beef meats and were grounded. Thus prepared raw meat balls were subjected to cooking at 100 ^{0}C for 90 min and autoclaved at 120 °C under 45-psi pressure for 2.5 h. All samples were prepared on three different days by three independent analysts.

4.3 Result and Discussion

4.3.1 DNA isolation and PCR optimization

DNA was extracted from raw and thermally treated meat samples using spin column based extraction technique which is known to reduce DNA loss during aqueous and organic phase separation (Karabasanavar et al., 2011). The highest DNA yield was obtained from oven heated samples (320-415ng/mg) in which number of cell per unit weight of the tissue were increased due to thermal dehydration (Karabasanavar et al., 2011). The lowest yield was obtained from extensively autoclaved meat samples (90-102 ng/mg) in which DNA was significantly degraded due to prolonged heat and pressure treatment (Ali et al., 2011). The yield of DNA was higher (445-460 ng/mg) in admixed samples over those of meat balls (354-370 ng/mg). This was probably due to the complex pool of DNA from the complex matrices of meatball formulation. The purity of all DNA samples was 1.90–2.0 (A260/A280) in triplicates. Higher annealing temperature increases primer specificity and reduces nonspecific PCR amplification (Ali, Hashim, Mustafa, & Che Man, 2012; Wu, Hong, & Liu, 2009). Hence, through a repeated run of gradient PCR, an optimized higher annealing temperature (58 ^{0}C) was obtained for all PCR run used in this study.

4.3.2 Pair wise distance and phylogenetic analysis

Pair wise distance and phylogenetic tree was constructed using alignment analysis of the 100 bp region of the cytb gene obtained from the sequencing of the PCR products under various conditions. The consensus gene sequences including primer binding sites were aligned with the tested 11 animal and 3 plant species. Both pair wiese distance obtained using maximum composite likelihood method (Tamura, Nei, & Kumar, 2004; Tamura et al., 2011) and phylogenetic tree built by Neighbor-Joining

method (Saitou & Nei, 1987) showed close relationship of dog with sheep (Figure 4.2 and Figure 4.3). However, the probability of non specific amplication of closely related sheep DNA was eliminated by the presence of mimimum 5 to 7 mismatched in the reverse and forward primer binding sites (Ali et al., 2012).

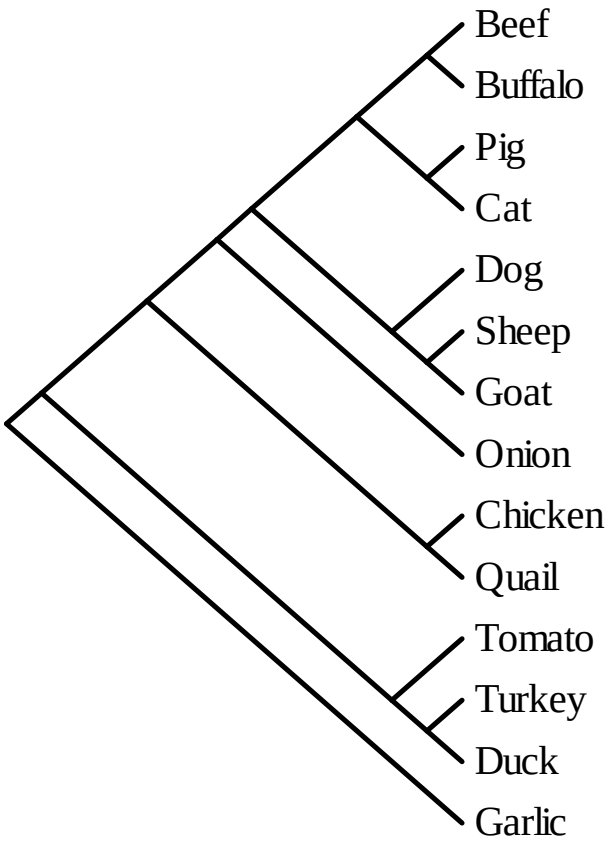

Figure 4.2 Evolutionary distance tree built from the 100 bp region cytb/cob-gene sequences of dog and 13 animal and plant species using Neighbour-Joining method for meatball analysis.

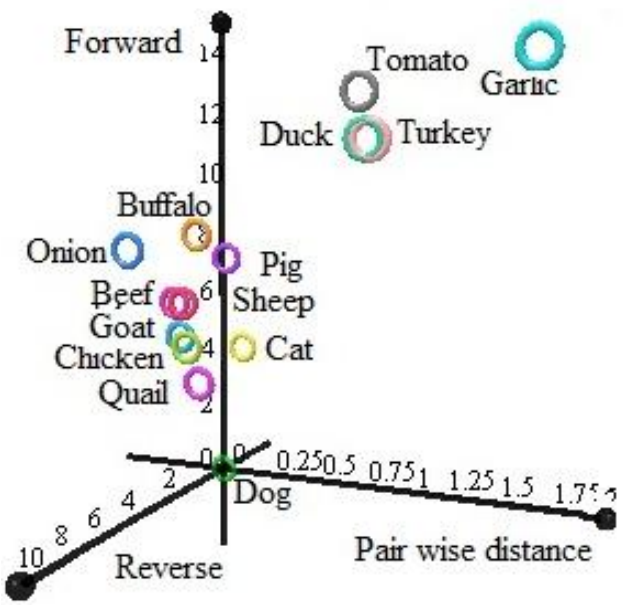

Figure 4.3 3D plot showing the strength of primers and target site for discrimination of dog. Here X axis represent pair wise distance, Y axis number of forward and Z axis number of reverse primer mismatch between canine and other potential animal and plant species for meat ball formulation.

4.3.3 Canine specificity under pure states

The designed primers were aligned against cytb/cob gene sequences of total 14 species including 9 halal, 2 non-halal and 3 plants species to find the distinct specificity of the primers at theoretical level (Data Supplementary Table CS-4.1). The primer pairs were further checked against other animal and plant species using BLAST search in NCBI data base. In both cases, 100% identity was found only with canine cytb gene. In real PCR run, the assay amplified only 100 bp canine cytb gene target (Figure 4.4). Sequencing of PCR product also confirmed the specificity for canine (Canis lupus familiaris) cytb gene.

It has been quoted in several reports that the efficiency of the PCR assay might reduce or end up with PCR amplification because of the presence of single mismatches in the primer binding region (Ali et al., 2012; Smith, Vigilant, & Morin, 2002; Wu et al., 2009). The proposed mt-cytb based primer pair had mutiple mismatches (>5 nucleotide) in the primer binding sites with the other tested species and in a real PCR run confirmed 100 % specificity only with the canine species. Previously, different species specific PCR assays using mitochondrial whole genome (322 bp) (İlhak & Arslan, 2007), mitochondrial cytb (808 bp) (Abdel-Rahman et al., 2009), D-loop (213 bp) (Gao et al., 2004) and 12S rRNA (101 bp) (Martín et al., 2007) have been proposed for dog meat detection. However, four of these assays used longer sized amplicon (≥213 bp) (Abdel-Rahman et al., 2009; Abdulmawjood, Schönenbrücher, & Bülte, 2003; Gao et al., 2004; İlhak & Arslan, 2007), which may not be stable under the extreme physio-chemical conditions of food processing. The latest studies appreciated short amplicon length PCR assay (<150bp) for the detection of species in highly processed foods (Ali et al., 2012; Rojas et al., 2010) . Martin et al (2007) proposed a short-amplicon length PCR assay targeting 101 bp region of canine 12S rRNA gene which should be suitable for the analysis of processed foods. However, the recent species identification schemes have showed that cytb-based PCR assays are more accurate to reconstruct mammalian phylogeny along with higher resolution over the other mitochondrial genes (Nicolas et al., 2012). This is probably because of the higher rate of intra-species conservation and protection of the mt-cytb gene over its counterparts. Therefore, we expected that a mt-cytb based short amplicon length (100 bp) PCR assay would detect canine derived materials under raw as well in highly processed commercial foods. This is probably because of the higher rate of intra-species conservation and protection of the cytb gene over its counterparts. Hence, we speculated

that mt-cytb based short amplicon length (100 bp) PCR assay would give better detection of canine derived materials from raw as well highly processed meatballs.

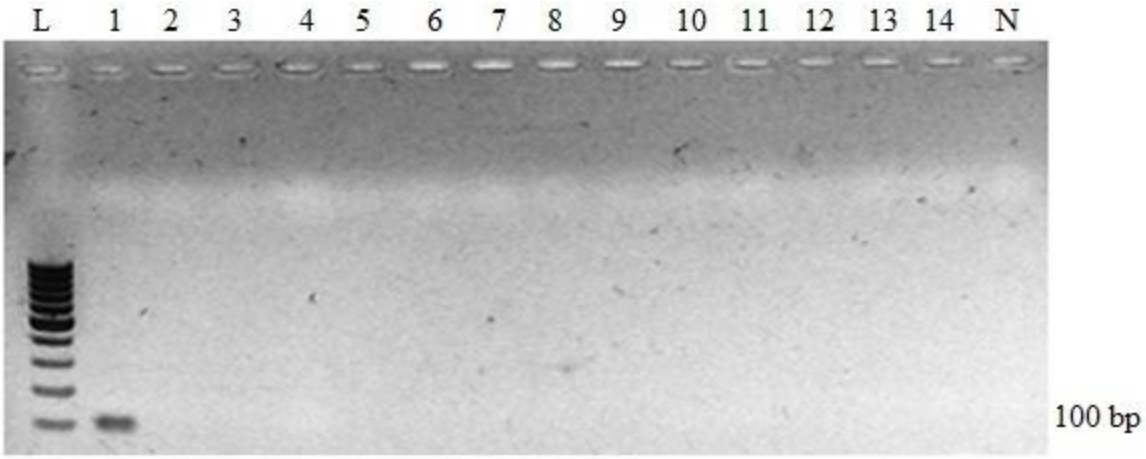

Figure 4.4 Species specificity analysis showing the 100 bp amplification of canine meat DNA; Lane 1: dog DNA, Lane 2-14: DNA from chicken, turkey, duck, quail, sheep, goat, beef, buffalo, pig, cat, tomato, garlic and onion respectively; lane N: Negative control.

4.3.4 Specificity and sensitivity in ternary meat mixture

Mt-cytb based canine specific PCR assay developed here was subsequently tested for the specificity and sensitivity under ternary admixed background composed of chicken meat and wheat flour contaminated with 1% to 0.01% of dog meat. The assay successfully detected canine specific target as low as 0.1% of dog meat (0.02 ng dog DNA) from contaminated admixtures of chicken and wheat flour (Figure 4.5), reflecting its high sensitivity and specificity even under complex background.

However, it has been mentioned in the previous chapter (Chapter 3) that sensitivity of the dog specific PCR assay documented by Martín et al. (2007) for a 101 bp fragment of 12S rRNA gene in meat-oats binary admixtures under normal

autoclaving condition was 0.1% (0.125 ng). Compared to this, our assay was 6.25 times more sensitive in terms of the amount of template DNA used (125 ng vs. 20 ng) and in terms of mixed meat matrices (ternary meat mixtures) under extensive autoclaving. For the detection of dog meat the highest detection limit (0.01%) was reported by Abdulmawjood et al. (2003) in a PCR-RFLP assay targeting a 808 bp fragment of mt-cytb gene. However, Abdulmawjood et al. (2003) did not mention how much DNA template they used. The percentage composition, which is an undefined method for concentration expression, can be manipulated through the use of higher amount of template DNA. Furthermore as illustrated earlier for extensive process food analysis, certain literatures reported for the better sensitivity and stability of the short DNA fragment based PCR assays over the longer ones (Ali, Hashim, Mustafa, & Che Man, 2012; Rojas, et al., 2010). Hence, the sensitivity of the PCR-RFLP assay documented by Abdulmawjood et al. (2003) cannot be higher than the present one and Martin et al.'s (2007).

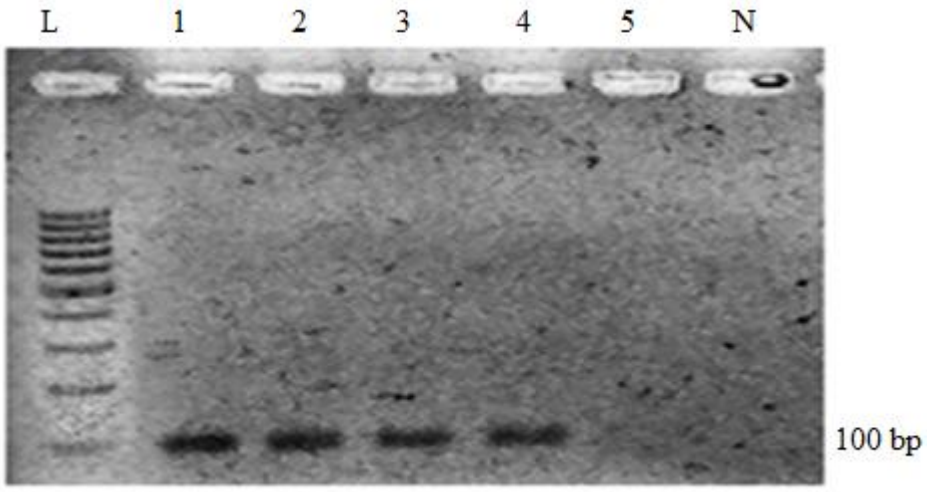

Figure 4.5 Specificity and sensitivity of canine specific PCR assay under chicken-dog-wheat flour ternary mixture. A 100 bp PCR product was found from 1 to 0.1% of dog meat contaminated ternary mixtures (Lanes 1-4) after extensive autoclaving. Lane L: 100 bp ladder; Lane 1-5: 1%, 0.5%, 0.2%, 0.1% and 0.01 % dog meat mixed with chicken and wheat flour respectively; Lane N: negative control.

4.3.5 Specificity and sensitivity under meatball matrices

Mince meat are often added in commercial food products (Tanabe, et al., 2007) and meat adulteration often takes place under mixed and processed conditions (Ali, Hashim, Mustafa, & Che Man, 2012). Therefore, we tested the performance of the assay under complex background of meatballs under different percentages of dog meat admixed condition with chicken and beef meatballs (1% to 0.01%). Thus prepared meatballs were further boiled at 100 °C for 90 min and autoclaved at 120 °C at 45 psi for 2.5 h to simulate cooking and extensive autoclaving. Clear 100 bp PCR products were observed from 1 to 0.2% of dog meat contaminated chicken (Figure 4.6, Lane 1-3, 6-8 &11-13) and beef meatballs. No PCR products were obtained from 0.1% and 0.01 % canine meat contaminated meatballs (Figure 4.6; Lane 4-5, 9-10 &14-15) and from negative control (Figure 4.6; Lane N).

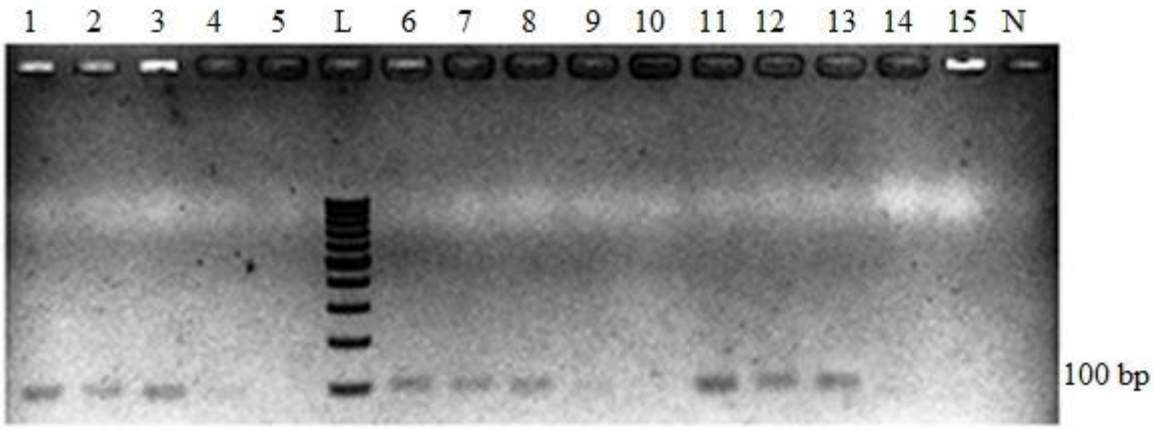

Figure 4.6 Specificity and sensitivity of canine specific PCR assay under meatball matrices. A 100 bp PCR product was obtained from 1 to 0.2% of dog meat contaminated chicken meatball under autoclaved, boiled and raw condition. Lane L: 100 bp ladder; Lane 1 to 5: autoclaved; Lane 6 to 10: boiled and Lane 11 to 15 : raw meatball samples contaminated with 1%, 0.5%, 0.2%, 0.1% and 0.01% dog meat, respectively; Lane N: negative control.

Thus 0.2% dog meat contaminated meatballs were used as a positive control for screening the halal logo containing commercial chicken and beef meatballs obtained from various super markets across Malaysia. In a blind experiment, five commercial brands (A-E) of chicken and beef meatballs were screen in triplicates on three different days against the 0.2% positive control. While all positive controls amplified the selective PCR products specific for canine cytb gene, no commercial samples were found to be positive (Table 4.2 and Figure 4.7).

According to previous literature all previously described PCR assays for canine meat detection were not tested for any commercial meat products (Chapter 3, Section 3.3.5). The presence of various additives and inhibitors in commercial meat and food products might prevent the primer binding at specific sites and reduce the amplification efficiency, diminishing the sensitivity and specificity of a PCR assay (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Calvo, Zaragoza, & Osta, 2001; Di Pinto, Forte, Conversano, & Tantillo, 2005). Therefore, we analyzed our assay performance in pure and contaminated state of commercial meatballs. A constant detection limit of 0.2% (0.04 ng DNA) was obtained in all meatballs positive control, demonstrating the performance of our PCR assay under the complex background of meatball matrices. In order to secure halal foods from production to storage, Department of Standards Malaysia has developed a halal standard (Talib, Ali, Anuar, & Jamaludin, 2008). Malaysian Government's goal is to make Malaysia as a halal hub in international market. (Talib et al., 2008).Therefore, we screen halal logo containing chicken and beef meatballs for dog meat adulteration from Malaysian local market. None of the samples displaying halal logo were found to be positive for dog meat. This reflects that the assay is applicable for halal authentication and trust worthy of the manufacturers.

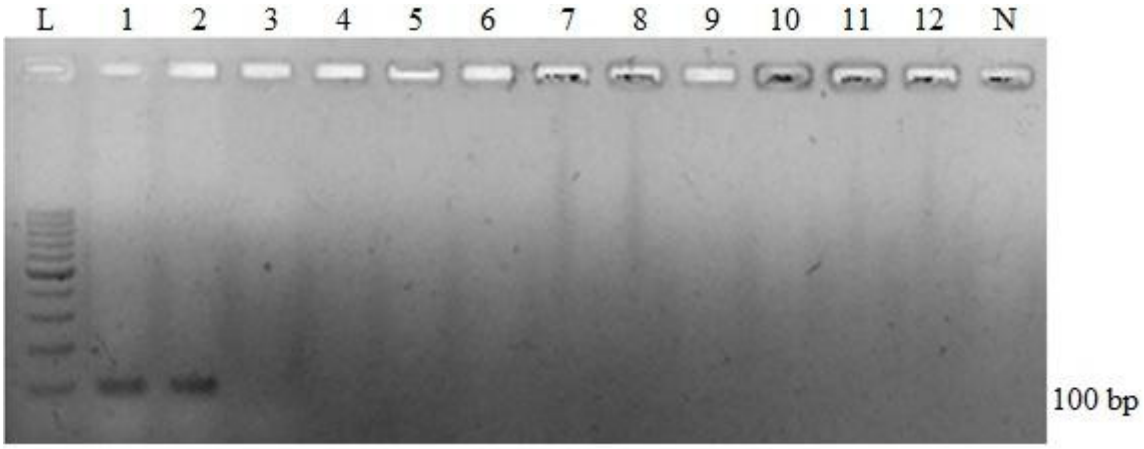

Figure 4.7 Screening of commercial meatballs using canine specific PCR assay. No PCR product was obtain from commercial samples. Lane L: 100 bp DNA Ladder; Lane 1-2: 0.2 % dog meat contaminated chicken and beef meatballs (positive control); Lane 3-7: commercial chicken meatballs (A-E); Lane 8-12: commercial beef meatballs (A-E) and Lane N: negative control.

Table 4.2 Meatball analysis using canine mt-cytb (100 bp) based PCR assay

Meatball sample	Day1	Day2	Day3	≥ 0.2 % Dog DNA Detection	Detection probability
Pure Chicken meatball	3	3	3	0/9	100
Pure Beef meatball	3	3	3	0/9	100
Pure Dog meatball	3	3	3	9/9	100
Dog meat contaminated with chicken	9	9	9	27/27	100
Dog meat contaminated with beef	9	9	9	27/27	100
Commercial chicken meatball					100
A	3	3	3	0/9	
B	3	3	3	0/9	100
C	3	3	3	0/9	100
D	3	3	3	0/9	100
E	3	3	3	0/9	100
Commercial beef meatball					
A	3	3	3	0/9	100
B	3	3	3	0/9	100
C	3	3	3	0/9	100
D	3	3	3	0/9	100
E	3	3	3	0/9	100

4.4 Conclusion

Here, a short amplicon length (100 bp) cytocrhome b based conventional PCR was documented for the detection of dog meat in commercial meatballs. The specificity test against 14 different species demonstrated that the assay was specific only for the dog cytb gene. Assessment under raw, ternary admixtures, meatball matrices along with cooking and extensive autoclaving condition, reflected that the method was suitable for the analysis of raw as well as processed meatballs. A blind experiment performed in triplicates on three different days by independent analysts, detected canine DNA only in positive controls. Screening of commercial meatballs from Malaysian supermarkets did not find any traces of canine tissues. The detection limit of the assay was 0.2% of dog meat (0.04 ng Dog DNA) from a complex DNA pool of meatball.

CHAPTER 5
POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF ADULTERATED CANINE MATERIALS IN KABSA & CURRY

(1st International Conference on Molecular Diagnostics and Biomarker Discovery and International Conference on Food Innovation 2014).

5.1 Introduction

5.1.1 Kabsa and curry

"Kabsa" is a traditional meat and rice dish. It is the most popular cuisine in Saudi Arabia and now extended its popularity from Arabian Peninsula to Malaysia (Midhet et al., 2010). Although kabsa belongs to halal food, individual consumer cannot verify the species by organoleptic test or even after consumption.

"Curry" is an Indian cuisine and a staple food in India. In the eighteenth century it was enjoyed by members of the East India Company living in Indian subcontinent (Maroney, 2011). Onward eighteenth and nineteenth-century, the greater metropolitan English families also adopted this Indian cuisine far from the Indian Territory and were exposed as dish through cookbooks. Now a days, over 10,000 Indian restaurants in the United Kingdom and Indian curry is one the most favourite dishes (Bharath, Sadiq, & Prema, 2007). The test and flavour of the Indian curry are extended all over the world including Malaysia where one of population group belongs to Indian ethnicity beside the native Malay and Chinese.

5.1.2 Current Meat Scandal in Kabsa and Curry

Time line	Products	Event	References
2014-04-18	Lamb curry	In UK, investigation of lamb curries in Teesside takeaways by Environmental Health and Trading Standards revealed that 41% of 29 lamb curry was found to be prepared/ admixed with beef.	Dale, 2014
2013-08-01	Meat	In Bahrain, a total of 172 frozen and canned meat brands were tested which were labelled as beef to sell at supermarkets and fast food chains by the Health Ministry in collaboration with the laboratories at the Arabian Gulf University. The test result showed that Six of them were contaminated with pork and 16 of them were contaminated with horsemeat.	Ali, 2013
2013-05-03	Lamb meat	In China, 20,000 tonnes of illegal meat products of fox, mink and rat was seized, which were in sale as mutton.	Kaiman, 2013
2013-03-28	Lamb curry	The random samples analysis from Indian curry and test of DNA from unnamed takeaway in UK, revealed that it is not contain any lamb, beef, chicken, pork, goat, horse or human tissue. Thus it lead the speculation that this "lamb" curry may contained dog or cat meat.	Sayid & Rossington, 2013

5.1.3 Prospect of Canine Species Detection in Kabsa and Curry

The history of pet animals revealed that dog was first domesticated around 15,000 years ago in East Asian countries when conventional farmed animals including cattle, horse, sheep, goat and chicken were not domesticated (Podberscek, 2009; Savolainen, Zhang, Luo, Lundeberg, & Leitner, 2002). Whatever might be the aim of domestication, dog meats have been consumed as a food by certain communities from ancient time to recent day (Podberscek, 2009; Vigne & Guilaine, 2004). Despite the state imposed banned and wide spread protests by animal right groups, dog meats have been consumed in Cambodia, Thailand, Vietnam, South Korea and China for decades.(Bartlett & Clifton, 2003; Podberscek, 2009). In Malaysia, reports have been made for dog meat consumption by foreign workers of Myanmar and Vietnam. In Islam, the dietary rules are strictly defined by Islamic law (Shari'ah) following the Quran (the divine book) and Hadith (the compilation of the traditions of Prophet Muhammad). The term "halal" is an Arabic word and it is ascribed to anything which is lawful or permitted for the Muslims. Meats of the domesticated split hoof animals such as cattle, buffalo, sheep, goat and camel are allowed for the Muslims but meats of the carnivorous animals like dog is totally prohibited in Islam (Khattak et al., 2011). Since there is no census data for dog population and stray dogs are available in many countries without any price, the probability of dog meat admixing particularly in more expensive halal meats cannot be ruled out.

Malaysia is a popular destination for tourist, and every year a lot of tourist either from Middle East and Indian subcontinent are here (Figure 5.1). The Muslim tourists from the Middle East prefer halal Arab cuisine and those from Indian subcontinent prefer Indian cuisine. However, the meat species in "kabsa" or in "curry "cannot be determined by visual inspection or simple organoleptic test. Hence to make forward the tourism industry in Malaysia and anatomical resemblance of dog and lamb it is

important to analyze these popular food items with proper laboratory protocol. Furthermore, human forensic evidences from crime scenes were often integrated with biomaterial of canine origin (Kanthaswamy, Premasuthan, Ng, Satkoski, & Goyal, 2012). Most of the conventional PCR assay for canine species detection used longer amplicon target (>150 bp) which have the drawbacks for highly degraded food or forensic sample analysis. Hence, for dog meat detection, we have developed a short amplicon based assay and tested its performance under certain meat products such as frankfurter and meatball (Chapter 3 and 4). Thus, the cytochrome b (cytb) based PCR assay with short-length amplicon size have shown its better stability and sensitivity. Therefore, for analyzing the meat adulteration in popular Arab (kabsa) and Indian cuisine (curry) we have utilized this short amplicon based canine specific PCR assay (100 bp) for the detection of canine (dog) meat in halal kabsa and curry.

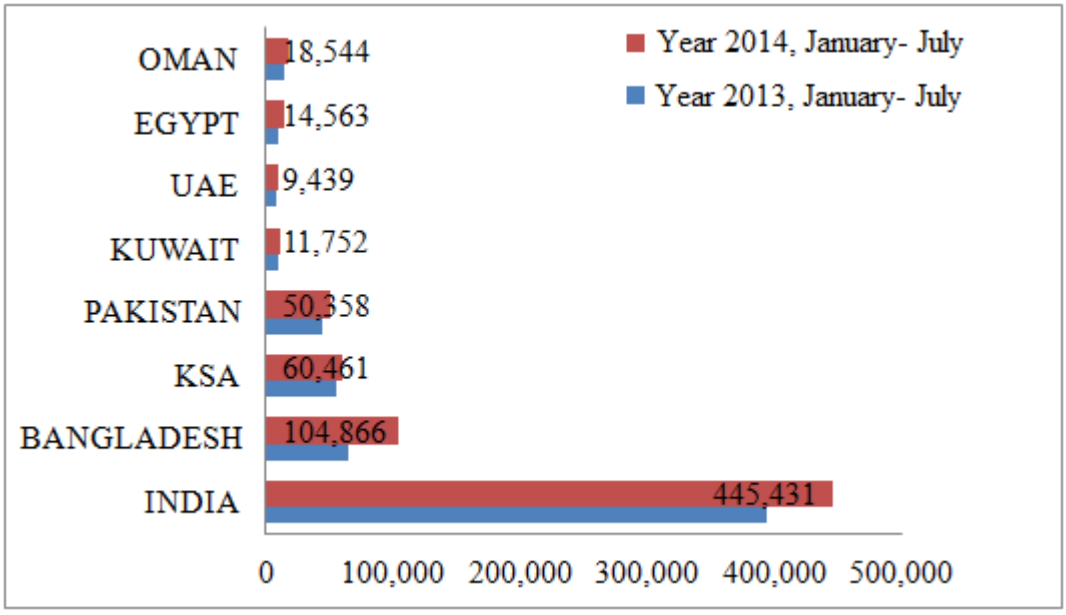

Figure 5.1 Tourist from Middle East and Indian subcontinent in Malaysia. (Source: Tourism Malaysia with the cooperation of Immigration Department, 2014).

5.2 Materials and Methods

5.2.1 Samples collection

Raw meat sample of 9 commonly used meat species (chicken, duck, turkey, quail, goat, sheep, beef, buffalo and pig) and 4 plant materials (rice, tomato, garlic and onion) were collected from various supermarkets located in three major Malaysian cities, namely, Serdang, Petaling Jaya and Kuala Lumpur in triplicates. The fresh typical dog meat samples were collected in triplicate from University Putra Malaysia and Jabatan Kesihatan Dewan Bandaraya Kuala Lumpur (DBKL) located at Air Panas in Kuala Lumpur. The collected samples were transported in an ice-box under chilled condition (4 ^{0}C) and were stored at -20 ^{0}C for further processing and DNA extraction. The samples of commercially available kabsa and curry were purchased in triplicates from 8 outlets across Malaysia.

5.2.2 DNA extraction and quality analysis

Total DNA, including mitochondrial and nuclear DNA, was extracted from 25 mg of raw meat samples of each species using NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel, Germany) following the manufacturer's instructions. For the processed and mixed admixtures, total DNA was extracted from 1 gm of specimen using adjusted volume of CTAB chemical (100 mg vs 1 gm), following the extraction protocol described in the chapter 3 sections 3.2.2. The quality of the extracted DNA samples were checked using 1% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, USA); by running in 1x LB buffer of pH 8.0 with a constant voltage of 170 V for 15- 20 min. DNA concentration and purity were measured by Eppendorf UV-vis Biophotometer (Eppendorf, Germany).

5.2.3 Canine specific biomarker

A pair of oligonucleotide primer (Forward 5′ CCTTACTAGG AGTATGCTTG 3′ and Reverse: 5′ TGGGTGACTGAT GAAAAAG 3′) specific for dog species were designed using the online version of the primer3plus software (www.bioinformatics.nl/cgi-bin/primer3plus/ primer3 plus.cgi). Primarily, the specificity of the designed primer-pair were screened for the unique canine specificity (species-specificity) and negative cross reactivity by local alignment tool "BLAST" (http://www.ncbi. nlm. nih.gov/blast). The mitochondrial cytb and apocytochrome b (cob) gene sequences of total 14 animals and plants including dog and human were retrieved from the published DNA sequences in National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm. nih.gov/). The retrieved sequences were aligned by publicly available mega 5 (Tamura et al., 2011) software to define the hyper variable region of the dog cytb gene with other species.

5.2.4 Specificity analysis

A two step method was followed to check the primers species specificity: (1) Primer design and oligonucleotide mismatch calculation in the primer binding site followed by pair wise distance analysis and construction of dendogram; (2) cross-species testing using DNA extracted from different potential species. To eleminate the probility of non target species amplification, 3D plot was created using Xlstat 2013 software, by calculating number of oligonucleotide mismatches in the primer binding site. For pair wise distance and construction of dendogram, 100 bp canine cytb gene sequence was aligned with the retrieved cytb gene sequences of 9 commonly available meat animals and cob gene sequences of 4 commonly used plant species in kabsa and curry preparation using ClustalW sequence alignment tool (Thompson, Higgins, & Gibson, 1994). The consensus regions of the gene were used to construct the

dendogram using Molecular evolutionary and phylogenetic analysis software, MEGA version 5 (Tamura et al., 2011). Cross-species testing were re-checked in a triplicate PCR experiment followed by visualization on agarose gel.

5.2.5 The PCR assay

Specific target region of dog mitochondrial cytb gene was amplified in a 20 µl of reaction mixture composed of 1x PCR master mix (Promega, Promega Corporation, Madison, USA) containing 50 units/ml of Taq DNA polymerase (supplied in a proprietary reaction buffer pH 8, 400 µM each dATP, dGTP, dCTP, dTTP and 3 mM MgCl2), 100 nM of each primer and 20 ng of total DNA. PCR was performed in a gradient thermocycler (Eppendorf, Germany) using an initial denaturation at 94 ^{0}C for 3 min followed by 30 cycles of denaturation at 94 ^{0}C for 30 s, annealing at 58 ^{0}C for 30 s and extension at 72 ^{0}C for 1 min. The final extension was performed at 72 ^{0}C for 5 min. The PCR products were stored at -20 ^{0}C for further analysis. The separation of PCR products, pre-stained with 6x loading dye, was performed in 1 % agarose gel (Promega, Madison, USA) in 1x LB buffer of pH 8.0 at a constant voltage of 170 V for 15- 20 min using 100 bp DNA ladder (Fermentas, USA). DNA visualization was performed using GelRed^{TM} (10,000X stock reagent into the agarose gel solution at 1:10,000) under a gel documentation system (AlphaImager HP; California, USA).

5.2.6 Meat sample processing

To assess the potentiality and applicability of the primer under various food processing conditions, the raw meats under pure state were subjected to different treatments after cutting into small pieces. To evaluate the cooking effects, the small cut frozen meat samples (100 g meat) were normalized at room temperature for each analysis and boiling was performed at 100 ^{0}C for 90 min. To evaluate the steam

cooking and autoclaving effects, meat samples were subjected to steam cooking at 100 ^{0}C for 45 min and autoclaved at 120 ^{0}C and 45 psi for 2.5h. The raw meat was also heated in an oven at 180 ^{0}C for 30 min to simulate the oven heating effect. The salting effects were studied after treating meat with 20% sodium chloride salt for 24 h at room temperature. All the treated meat samples were stored at -20 ^{0}C for further analysis.

5.2.7 Kabsa and curry preparation

Experimental pure lamb and dog meat kabsa were prepared following basic ingredient level described by Kaufman (2010). Briefly, meat pieces (100 gm) were browned in melted butter in non-stick frying pan at medium heat. Onions were added to the meat pieces and sautéed until translucent. Subsequently tomato, garlic, salt and spice admixture was added and heated to boil. After boiling, the temperature was reduced to low, covered and simmered 1 hr for lamb and dog kabsa. Finally, rice was added and cooked along with the meat pieces at low heat for 20 min. To stimulate the dog meat admixing in commercial lamb kabsa, 100 gm of lamb kabsa meat were spiked with 10%, 5%, 1%, 0.1%, and 0.01% of dog meat.

To evaluate the assay performance in complex background of Indian curry, 100g specimen of dog and lamb meat were cut into small cubes and added with salts and other spices as described else where to determine the canine meat adulteration (Rajkumar, Dushyanthan, & Das, 2010). Thus prepared dog and lamb meat with spices and salt were cooked for 90 min at 100^{0}C separately. After cooking meats were washed in running tape water and 100 g specimen of lamb meat were mixed with 10%, 5%, 1%, 0.1%, and 0.01% dog meats (w/w) to simulate dog meat adulteration in curry.

All admixed meat samples were subjected to vigorous blending to make a homogenous mixture and were prepared in three replicates on three different days. Samples were kept in -20 ^{0}C for DNA extraction and further analysis.

5.3 Results and Discussion

5.3.1 DNA extraction and PCR optimization

Spin column based DNA isolation kit was used for the extraction of DNA from each of the raw meat samples because of its high yield (Karabasanavar et al., 2011). A more sensitive CTAB method was applied to extract DNA from meat admixtures. The yield of DNA was higher in the oven heated samples (320-415ng/mg) over those of raw and untreated meat samples (180-230 ng/mg). This was probably because of the dehydration process which increases number of cell per unit weight of the tissue (Karabasanavar et al., 2011). The optimum PCR yield was found at 58 ^{0}C and thus all the subsequent PCR reactions were performed at this optimized annealing temperature.

5.3.2 Pair wise distance and canine species discrimination

For pair wise distance and construction of radiation tree, 100 bp consensus sequences were obtained from alignment of dog cytb gene sequence with total 13 animals and plant species potential in kabsa and curry preparation. Pair wise distance analysis using Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004; Tamura et al., 2011) showed a close relationship of dog cytb gene sequence with sheep (0.26). Radiation tree build by Neighbor-Joining method (Saitou & Nei, 1987) also showed a close relationship of dog cytb gene sequence with sheep (Figure 5.2). However, calculation of oligonucleotide mismatch (5-15) and creation of 3D plot re confirmed the primers property with clear dicrmination of the dog from all the tested species in this experiment (Figure 5.3).

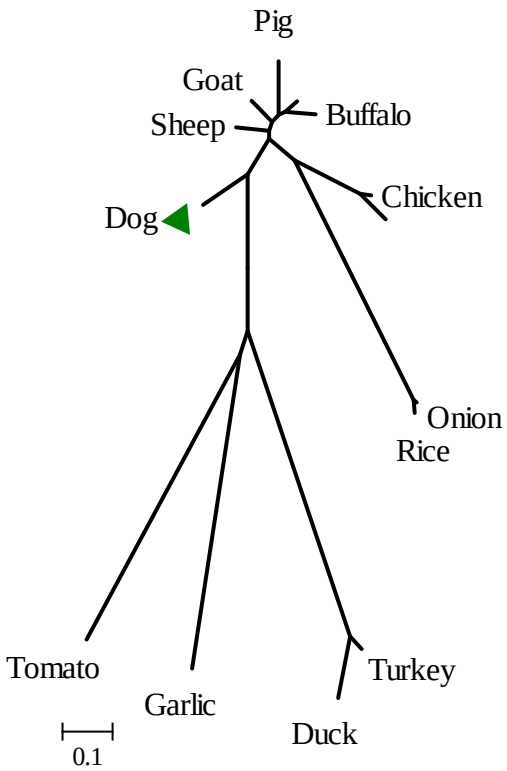

Figure 5.2 Radiation tree by Neighbor-Joining method showing the distances between 100 bp cytb gene sequence of dog and other 13 potential animals and plant species potential for kabsa and curry preparation.

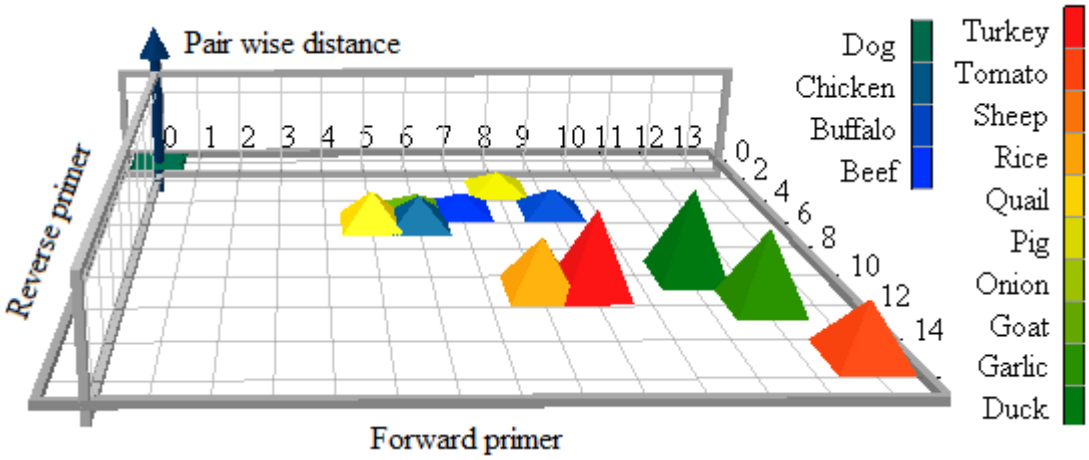

Figure 5.3 3D plot showing the discrimination properties of the canine specific primers using number of forward and reverse primers oligo nucleotide mismatch to prevent non specific amplification of other commonly available 13 meat and plant species for kabsa and curry preparation.

5.3.3 Canine Specific PCR assay

Species-specific PCR techniques are widely used for the detection of different raw meat species and admixed meat products, such as beef (Mane, Mendiratta, & Tiwari, 2012), buffalo (Mane, Mendiratta, Tiwari, & Bhilegaokar, 2012), ostrich (Colombo, Viacava, & Giaretti, 2000), pork (Ali, Hashim, Mustafa, & CheMan, 2012), chicken and poultry (Stamoulis, Stamatis, Sarafidou, & Mamuris, 2010). However, the presence of mismatches in the primer binding region significantly reduces the efficiency and specificity of a PCR assay, causing cross-species amplification or amplification failure in some instances. Therefore, we confirmed canine specificity of the designed primer pairs both the theoretical and experimental levels. The theoretical specificity was checked by alignment analysis of the amplicon region of the dog cytb gene (Canis lupus familiaris: JF489119.1) with those of 9 commonly available meat animal species (Chicken/Gallus gallus: EU839454.1, Duck/Anas platyrhynchos: HQ122601.1, Turkey/Meleagris gallopavo: HQ122602.1, Quail/Coturnix coturnix: EU839461.1, Sheep/Ovis aries: EU365990.1, Goat/Capra hircus : EU130780.1, Beef/Bos taurus: EU807948.1, Buffalo/Bubalus bubalis: D32193.1, Pig: GU135837.1) and apocytochrome b (cob) gene of 4 plant species (Rice: X53710.1, Tomato: XM004251454.1, Garlic: AF356823.1, Onion: GU253304.1) retrieved from the NCBI database, using multiple sequence alignment tools. The alignment analysis revealed perfect matching only with the canine cytb gene and multiple mismatching with the other species in the primer binding regions (≥ five nucleotides). The primers' sequences were further blasted in the NCBI data base against non-redundant sequences to eliminate the probability potential cross species amplification. Finally, the practical aspect of the primer specificity was confirmed through a real PCR reaction with 14 different animal and plant species usually used in kabsa or curry preparation. A 100 bp PCR product was found only from dog meat DNA. No PCR product was obtained from

other meat DNAs, eliminating the probability of any cross-species amplification (Figure 5.4).

As describes in earlier chapters in different sections (Chapter 2,3,4), previously dog meat was detected using PCR assays which targeted mitochondrial genome (İlhak & Arslan, 2007), mitochondrial cytb (Abdel-Rahman, El-Saadani, Ashry, & Haggag, 2009), D-loop (Gao, Xu, Liang, Zhang, & Zhu, 2004) and 12S rRNA gene (Martín et al., 2007 (Chapter 3, Section 3.3.5 & Chapter 4, Section 4.3.5). The PCR amplicon size for dog specific mitochondrial genome, mitochondrial cytb and D loop gene were 322 bp (İlhak & Arslan, 2007), 808 bp (Abdel-Rahman et al., 2009) and 213 bp (Gao et al., 2004) respectievly. However, recent studies demonstrated that short amplicon length (<150bp) PCR assays are more appropriate over the longer one for the analysis of processed food products (Rojas et al., 2010). In line with this assumption, a short length (101 bp) PCR assay was proposed targeting 12S rRNA gene (Martín et al., 2007). It has been reported that cytb-based PCR assays are more appropriate for accurately reconstructing the mammalian phylogeny and provide better resolution in species identification. (Nicolas et al., 2012) Hsieh and other reported that mt-cytb gene is more useful for detecting species origin in degraded forensic specimen.(Hsieh et al., 2001) Moreover, an extraordinary stability of a cytb based pork specific (109 bp) PCR assay in extensively processed meat products was described by Ali et al., (2012). These prompted us to use cytb based short amplicon length (100 bp) PCR assay for the identification of canine origin materials in lamb kabsa and curry.

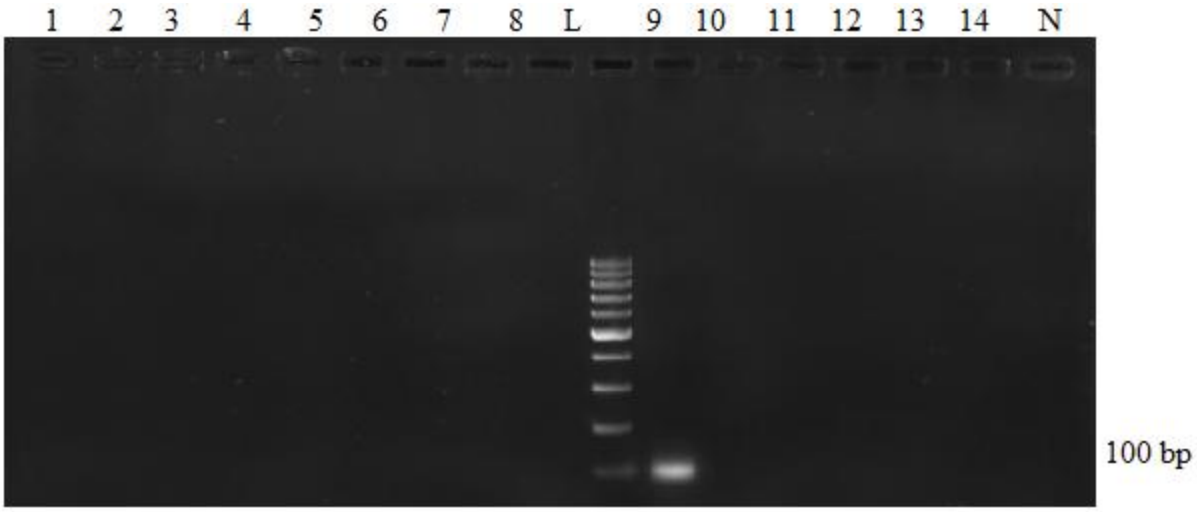

Figure 5.4 Specificity test of canine specific primers using DNAs from dog and commonly available animals and plant species DNAs usually used in kabsa or curry preparation. Legends: Lane 1: Chicken DNA; Lane 2: Duck DNA; Lane 3: Turkey DNA; Lane 4: Quail DNA; Lane 5: Beef DNA; Lane 6: Buffalo DNA; Lane 7: Goat DNA; Lane 8: Sheep DNA; Lane L: 100 bp ladder; Lane 9: Dog DNA; Lane 10: Pig DNA; Lane 11: Onion DNA; Lane 12: Garlic DNA; Lane 13: Tomato DNA; Lane 14: Rice DNA and Lane N: Negative Control.

5.3.4 Stability of canine specific DNA template

To verify the stability of template DNA under various food processing conditions, the canine specific PCR assay was performed with 20 ng total DNA extracted from raw, boiled, autoclaved, steam cooked, oven heated and salt treated dog meat samples. A 100 bp PCR product was obtained from all samples (Figure 5.5), reflecting the stability of the canine cytb template under potential meat processing conditions.

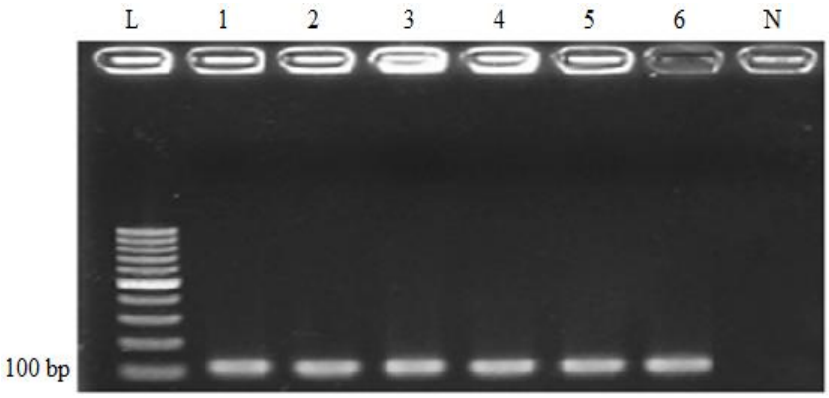

Figure 5.5 The efficiency of dog specific PCR assay under different food processing conditions. Legends: Lane L: 100 bp Ladder; Lane 1: Fresh meat; Lane 2: Autoclaved meat; Lane 3: Boiled meat; Lane 4: Oven heated meat; Lane 5: Steam cooked meat; Lane 6: salt treated meat; and Lane N: Negative Control.

Martín et al., (2007) documented PCR assay that targeted 101 bp amplicon of canine 12S rRNA gene, was stable under various autoclaving conditions such as at 120 °C for 50 min, 110 °C for 120 min, and 133 °C for 20 min under 45 psi autoclaving pressure (Martín et al., 2007). Previous studies demonstrated breakdown of 411bp target of 12S rRNA gene under extensive autoclaving under admix condition (Ali et al., 2011), but a 109 bp target of cytb gene was stable (Ali et al., 2012). Therefore, successful amplification of 100 bp cytb gene target in this study after prolonged autoclaving of dog meat (2.5 h) reflected an extraordinary stability of the PCR assay. To stimulate the meat kebab and meat patty preparation, the meat samples were oven heated 180 °C for 30 min. No adverse effect in PCR products were observed with DNA extracted from oven-heated sample. For food safety 20% common salt are used to inhibit certain bacterial growth (Tiganitas, Zeaki, Gounadaki, Drosinos, & Skandamis, 2009). We tested the dog meat with 20% common salt and the assay successfully amplified the target. Thus, we found that the developed assay was stable under all potential cooking and processing conditions. This was probably because of very short amplicon length and stability of cytb gene.

5.3.6 Dog meat detection in kabsa and curry

Finally, the assay performance was tested in popular lamb kabsa and curry. The dummy kabsa were prepared in pure as well in 0.01% to 10% dog meat spiked state. 100 bp Clear PCR products were obtained from pure and 0.1% to 10% dog meat spiked dummy lamb kabsa and curry (Figure 5.6 a and b). Thus, 0.1% dog meat spiked dummy kabsa and curry with 100% detection probability (Figure 5.7) were used as a positive control to screen commercial lamb kabsa and curry obtained from different 8 different outlets of Malaysia. No commercially served kabsa or curries from any Malaysian outlets were found to be admixed with dog meat (Table 5.1, Figure 5.7).

Previously proposed different PCR assays for canine meat detection (Abdel-Rahman et al., 2009; Abdulmawjood, Schönenbrücher, & Bülte, 2003; Gao et al., 2004; İlhak & Arslan, 2007; Martín et al., 2007) were not tested for kabsa or curry analysis. . The presence of various additives and inhibitors in commercial meat and food products may prevent the primer binding at specific site and reduce the amplification efficiency of a PCR assay (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Calvo, Zaragoza, & Osta, 2001; Di Pinto, Forte, Conversano, & Tantillo, 2005). Therfore, we had developed and tested the short amplicon based canine specific assay targeting mt-cytb gene (Chapter 3 and 4). The assay was proven for a high specificity and sensitivity. Therefore, we analyzed this cytb based assay performance for lamb kabsa and curry. A constant detection limit of 0.1% (0.02 ng DNA) was obtained in all positive control, repeated the high performance of the PCR assay. It may due to the shorter size of the cytb gene target, which is known for better sensitivity and stability under harsh conditions.

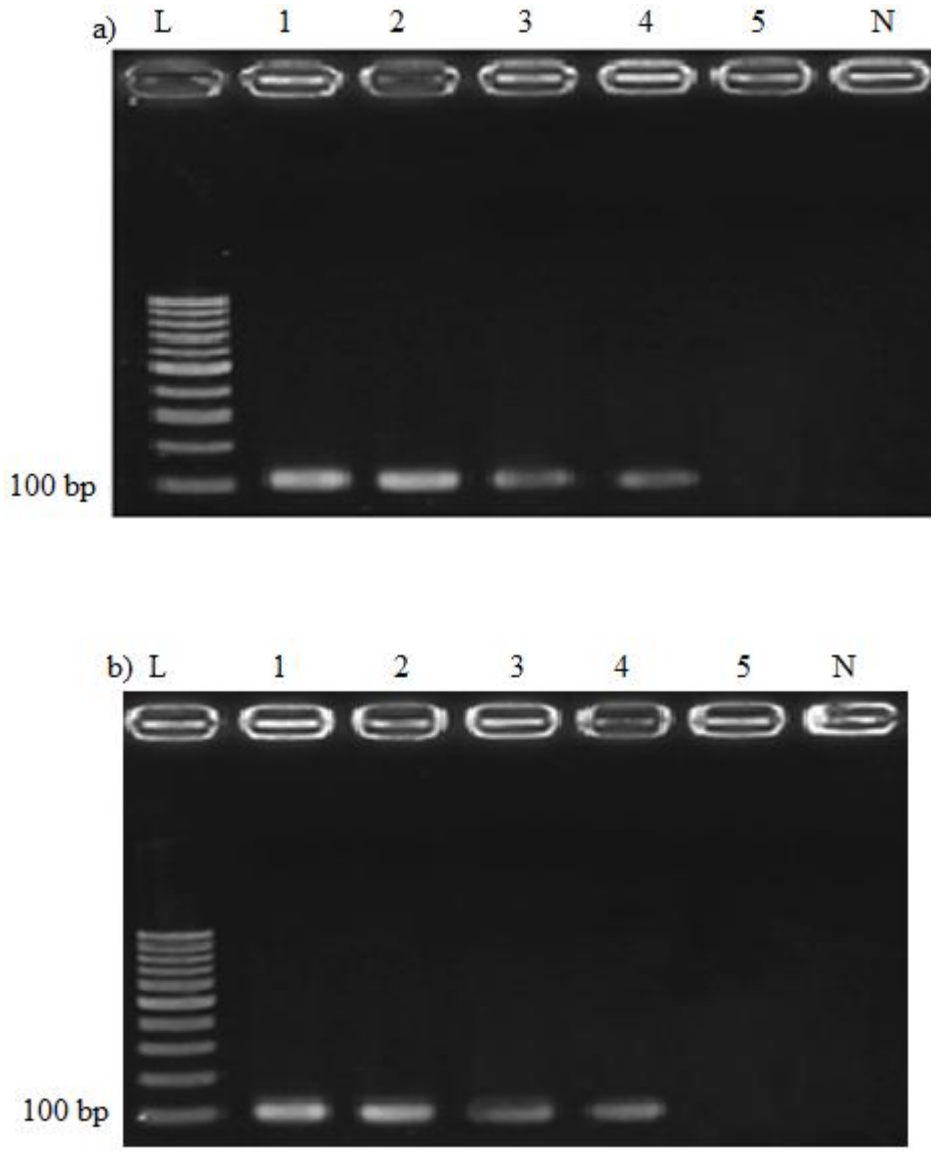

Figure 5.6 The detection limit (10% to 0.1%) of canine specific PCR assay using kabsa and curry samples with agarose gel electrophoresis. In b) Lane L: 100 bp Ladder; Lane 1 to 5: Lamb kabsa mixed with 10%, 5%, 1%, 0.1% and 0.01% dog meat respectively and Lane N: Negative Control, In b) Legends: Lane L: 100 bp Ladder; Lane 1 to 5: Curry with 10%, 5%, 1%, 0.1% and 0.01% dog meat respectively; Lane N: Negative Control.

There is large influx of tourist from Middle East and Indian subcontinent coming to Malaysia (Figure 5.1). Arabs from the Gulf region are travelling with their spouse and children to spent vacation and they prefer “Arab cuisine” (Ibrahim, Zahari, Sulaiman, Othman, & Jusoff, 2009). Furthermore, the tourist from Indian subcontinent prefer Indian dish and popular of which is meat curry as it is the part of staple food (Maroney, 2011). Beside the Indian ethnic Malaysian and tourist, the Muslim population of Malaysia also prefers either Arab or Indian cuisine Halal dishes. Therefore, we found the validity and applicability of this PCR assay for Halal status detection of kabsa and curry from 6 different outlets across Malaysia where none of the samples were found positive. It reflects the validity of the Halal status of “Arabic or Indian cuisine” in Malaysia in terms of canine meat admixing.

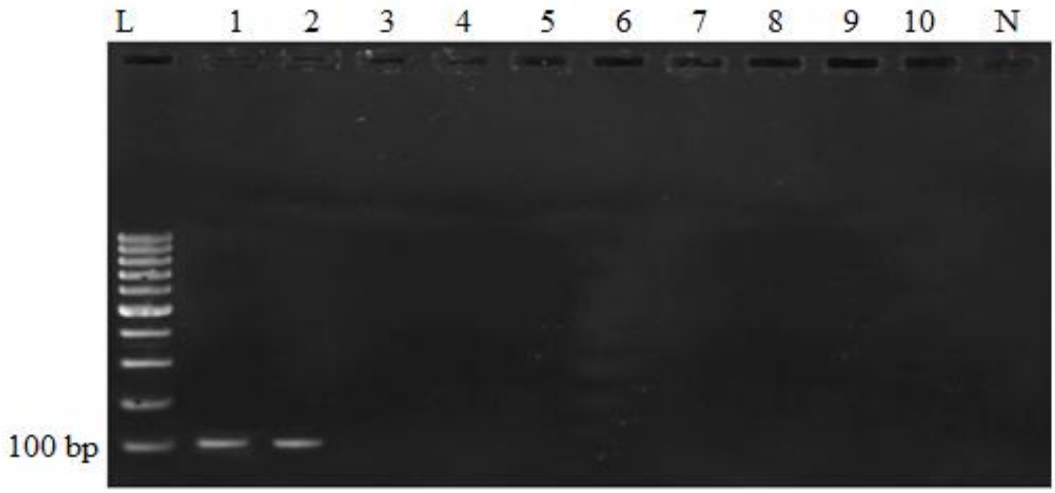

Figure 5.7 Commercial Kabsa and analysis showing 100 bp PCR products from 0.1% dog meat admixed lamb kabsa and curry. No PCR product from any commercial kabsa or curries from different Malaysian outlets were found positive for canine species. Legends, Lane N: Negative control; Lane L: Ladder; Lane 1 and 2: 0.1 % dog meat contaminated lamb kabsa and curry; Lane 3 to 6: Commercial kabsa (A-D); Lane 7 to 10: Commercial curry (A'-D').

Table 5.1 Analysis result of different kabsa and curry samples for dog meat detection using canine specific mt-cytb (100 bp) based PCR assay.

Samples	Dog meat (%)	Day 1	Day 2	Day 3	Positive detection	≥ 0.1 % dog meat positive samples	Detection probability (%)
Dog meat admixed Kabsa	10	3	3	3	9/9	27/27	100
	1	3	3	3	9/9		
	0.1	3	3	3	9/9		
	0.01	3	3	3	0/9	-	
Commercial kabsa	-	3	3	3	0/9		
Dog meat admixed Curry	10	3	3	3	9/9	27/27	100
	1	3	3	3	9/9		
	0.1	3	3	3	9/9		
	0.01	3	3	3	0/9	-	-
Commercial curry	-	3	3	3	0/9		

5.4 Conclusion

The canine specific PCR assay suitable for authenticating canine tissues under various food processing conditions was tested for its application in Arab and Indian cuisine analysis. The assay was tested under various food processing conditions as well as in kabsa and curry and found to be suitable for detecting as low as 0.1% dog meat admixture under complex food matrices. No positive amplification of canine specific cytb gene target any of the commercial samples of kabsa and curry collected from different outlets of Malaysia showed the validity of the “Halal” status of these popular foods.

CHAPTER 6
LAB-ON-A-CHIP PCR-RFLP ASSAY FOR THE DETECTION OF CANINE DNA IN BURGER FORMULATIONS

(Food Analytical Methods; 2015, DOI: 10.1007/s12161-015-0090-1)

6.1 Introduction

6.1. 1 Burger

Burger is a sandwich consisting of a bun, a cooked patty prepared by mixing emulsified ground meat of chicken, beef, lamb, pork or fish with certain ratios of starch, seasonings, and salts. It is often served with condiments such as mayonnaise, mustard, ketchup and relish along with lettuce, tomato, bacon, onion, pickles, cheese (Kenda, 1990). In recent trends, consumers in their busy life prefer fast, readymade and easy foods. Burger is one of the popular ready to serve food items to full this purpose (Lawrie & Ledward, 2006). Burger is a fast growing ready to eat food and the sales for the year 2013 was a total of 121.77 billion U.S. dollars from world leading burger chains (Figure 6.1) (Information Resources, 2013).

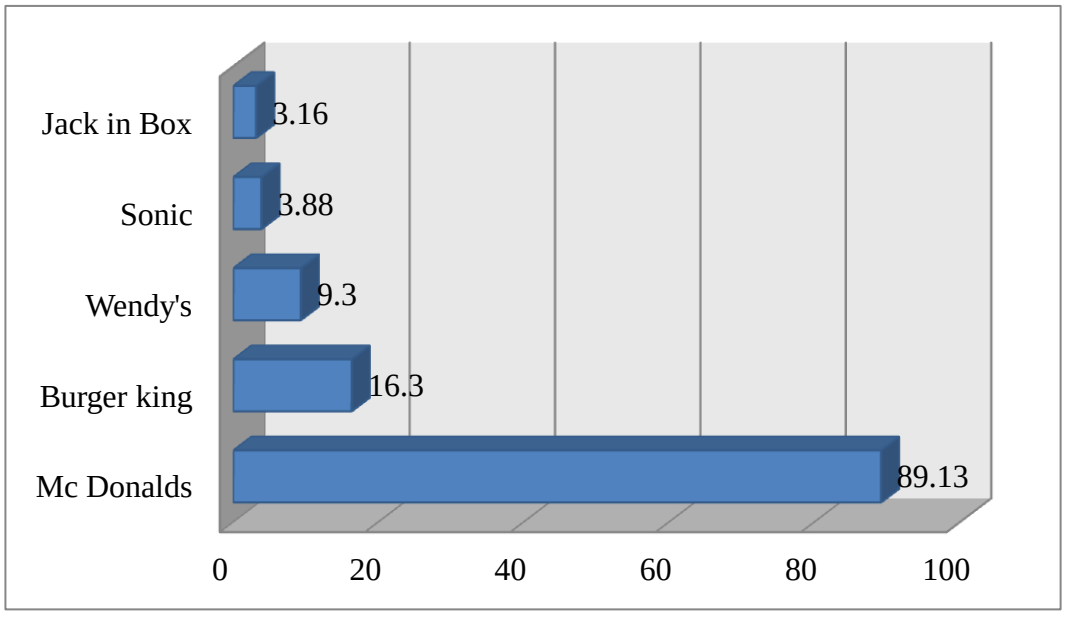

Figure 6.1 Sales of the leading burger chains worldwide in 2013 (in billion U.S. dollars) (Information Resources, 2013).

6.1.2 Current Burger Meat Scandal

Date	Product	Event	References
2013-03-28	Beef burger	In UK, bovine blood, chicken scraps and a high level of chicken blood was found from beef burger analysis from a fast food shop.	Chatterji, 2013
2013-03-26	Chicken nuggets	In Greece, horse meat had been found in chicken nuggets.	Embiricos, 2013
2013-03-14	Burger	Burgers and lasagne saled in Beefeater, Brewers Fayre, Table Table, Taybarns and Premier Inn hotels were found to be contaminated with horse meat. Furthermore, the beef burger served for hospital patient was containing horse meat.	Webb, 2013
2013-02-25	Beef burger and sausages	In South Africa, meat of water buffalo, goat and donkey was found in various meat products such as beef burger and sausages	Janice & Jaco, 2013
2013-01-15	Frozen beef burger	The Food Safety Authority of Ireland (FSAI) announced that horse meat had been found in frozen beef burgers at several Irish and British supermarkets, including Tesco, Asda, Dunnes Stores, Lidl, Aldi and Iceland.	Carty, 2013; Reilly, 2013

2013-01-11	Burger	Quantitative analysis of the 10 burger samples from Tesco made by Silvercrest showed 1 with 29% level of equine (horse) DNA.	Reilly, 2013
2012-12-07	Burger	Irish authorities detected 29% horse meat content in the beef burger from ABP Food Group.	Fionnan & Aideen, 2013
2012-11-30	Salami, beef meal product, burger	The result of the quantitative test revealed 85% beef burger products were positive for porcine (pig) DNA and 37% beef burger products were positive for equine (horse) DNA. Test result of the burgers came from 6 plants in Ireland and 3 plants in the UK were positive for equine DNA came from 2 plants in Ireland and one from the UK.	Reilly, 2013

6.1.3 Prospect of Current Lab-On-A-Chip Based PCR-RFLP Assay

Despite of the religious taboo and huge protection from animal right group, dog meats have been reported for human consumption in many parts of the world including South Korea, China and Vietnam (Podberscek, 2009). Availability of stray dogs in certain countries (Kumarapeli and Awerbuch-Friedlander, 2009; Totton et al., 2010) make dog meat as potential source for fraudulent admixing with costly meats to get extra profit.

The microfluidic-based lab-on-a-chip technology provides more effective way for PCR endpoint detection. This is an easy user friendly analysis method integrating capillary electrophoresis (CE) in chip (Lab on a Chip). High resolution and speed with

better reproducibility using less reagents and samples, facilitates CE-based techniques over the traditional agarose gel electrophoresis (Funes-Huacca et al., 2004). Furthermore, the automated Lab on a chip for DNA detection lacks analyst exposure to hazardous chemicals. The Lab on chips loading wells are fabricated with small single unit with etched capillaries. The fragments of DNA separated by CE are detected by laser-induced fluorescence analyzer. This method utilizes disposable chip and comparatively lower cost instrumentation than Real time or DNA sequencing. Therefore, it is getting wider acceptance in food analytical laboratories and also been used for meat species detection (Dooley & Garrett, 2001; Dooley et al., 2005).

We have described elaborately the utilization of the PCR-RFLP assay for different food species detection in the literature review (Chapter 2.6.3.6.). However for canine species detection the previously described PCR-RFLP assay had longer amplicon size which have drawback for process food analysis. All the previously documented PCR based assays for canine species detection were based on traditional gel electrophoresis for PCR end point detection. There were a lot of meat adulteration reports for this popular minced meat product throughout the world (Chapter 6.1.2). However, none of the previous assay was tested under the commercial burger backgrounds which have huge market value (Figure 6.1). Therefore, in the present research we have utilized the short amplicon based canine-specific PCR and RFLP assay for detection of dog DNA in raw and process food such as burger. The assay was based on lab-on-a-chip based endpoint detection technique and was proven under model experiment of different admixed conditions as well as under more complex DNA pools of burger formulation.

6.2 Materials and Methods

6.2.1 Samples collection

Raw meat samples of chicken (*Gallus gallus*), quail (*Coturnix coturnix*), sheep (*Ovis aries*), goat (*Capra hircus*), beef (*Bos taurus*), buffalo (*Bubalus bubalis*), pig (*Sus scrofa*), cucumber (*Cucumis sativus*) and tilapia (*Oreochromis niloticus*) were purchased in triplicates from the various wet markets in Kuala Lumpur and Selangor in Malaysia. Dog (*Canis lupus*) meat samples were collected from three different dogs, officially killed by Dewan Bandaraya Kuala Lumpur (DBKL) located in Taman Air Panas in Kuala Lumpur as a measured of population control. Commercial samples of eight halal branded chicken and beef burgers were purchased in triplicates from eight different outlets across Malaysia. All samples were transported under ice chilled condition (4 ^{0}C) and were stored at -20 ^{0} C for DNA extraction and future work.

6.2.2 DNA extraction

DNA was extracted from 25 mg of raw meat samples using NucleoSpin® Tissue extraction kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. For admixed and burger samples DNA was extracted from 1 gm sample using increased amount CTAB reagent following the extraction protocol as described in the chapter 3 sections 3.2.2. For good quality DNA it was further purified using Promega Wizard™ DNA isolation kit (Promega Corporation, Madison, USA). The concentration and purity of the DNAs were determined using UV-vis spectrophotometer Biochrom Libra S70 (Biochrom Ltd., UK).

6.2.3 Canine specific Primer

A pair of canine specific primers (Forward 5′ CCTTACTAGGAGTATGCTTG 3′ and Reverse: 5′ TGGGTGACTGATG AAAAAG 3′) were designed using online version of primer3plus software (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) targeting a 100 bp fragment of canine cytb gene (GenBank AF034253.1) which included a 30-bp *Alu*I-cut site as internal oligo. The canine specificity of the primers was theoretically determined using NCBI blast analysis (www.ncbi.nlm.nih.gov). Furthermore, cytb gene sequences of 13 meat-providing animals and fish species (Chicken: EU839454.1, Duck: HQ122601.1, Turkey: HQ122602.1, Quail EU839461.1, Sheep: EU365990.1, Goat: EU130780.1, Beef: EU807948.1, Buffalo: D32193, Pig: GU135837.1, Tuna: AM989973.1, Sardine: DQ197989.1, Tilapia: AF015020.1, Prawn: AF125382.1) and apocytochrome (cob) gene sequences of 6 plant (Tomato: XM004251454.1, Onion: GU253304.1, Wheat: AF337547.1, Maize: X00789.1, Potato: X58437.1, Cucumber XM004153108.1) were retrieved from publicly available NCBI database and were aligned by ClustalW multiple-sequence alignment tool (Thompson, Higgins, & Gibson, 1994). The consensus canine specific 100 bp cytb gene sequence was used to study pair wise distance and construct dendogram using molecular evolutionary and phylogenetic analysis software, MEGA version 5 (Tamura et al., 2011). Primers, *Alu*I cut site and number of oligonucleotide mismatch in the primer binding sites were analysed by BioEdit software version. 7.2. A 3D plot was created based on pair wise distances of 100 bp canine specific site and number of oligonucleotide mismatches by XLSTAT software (Addinsoft, 2013) to define the potentiality of the primers to specifically amplify the 100 bp fragment of canine cytb gene. For internal control, a previously designed eukaryotic primer pair (Forward 5′ GGT AGT GAC GAA AAATAA CAA 3′ and Reverse 5′ ATA CGC TAT TGG AGC TGG AATTA C CTAC AGG AC3′)

targeting 141-bp conserved fragment of eukaryotic 18S rRNA gene were used (Rojas et al., 2010). All primers were obtained from the 1st BASE Laboratories, Pte. Ltd. in Selangor, Malaysia and were tested in a real-PCR run.

6.2.4 PCR Amplification

We amplified a 100 bp canine and 141 bp eukaryotic targets using a PCR assay of 20µl reaction volume composed of 5X colorless GoTaq Flexi buffer supplied with the enzyme, 1.25 µ GoTaq Flexi DNA Polymerase, 1.25 mM MgCl2, 200 µM dNTP's (Promega, Madison, USA), 0.4 µM each primer and 20 ng of total DNA extracted from each sample. The cycling conditions used in a gradient thermocycler (Eppendorf, Germany) were initial denaturation at 94 ^{0}C for 3 min followed by 30 cycles of denaturation at 94 ^{0}C for 30 s, annealing at 58^{0}C for 30 s and extension at 72 ^{0}C for 1 min. The final extension was performed at 72 ^{0}C for 5 min. PCR products were detected on a microfluidic-based lab-on-a- chip using Experion DNA 1K analysis kit following manufacturer's instruction (Bio-Rad Laboratories, USA).

6.2.5 Preparation of binary admixtures

To evaluate the performance of the PCR assay in meat products, binary admixtures of dog-chicken and dog-beef were prepared by spiking 10%, 1%, 0.1% and 0.01% dog meat in chicken and beef in a 100 g specimen (Ali, Hashim, Mustafa, & Che Man, 2012). Thus, prepared admixtures were blended vigorously to make a homogeneous admixture. All admixtures were prepared on three different days by three independent analysts and were autoclaved at 120 °C under 45-psi pressure for 2.5 h before extracting DNA.

6.2.6 Burger preparation

To simulate commercial burger, model raw, autoclaved and ready to eat burgers were prepared using dog, chicken and beef meat following (Ali, Hashim, Dhahi, et al., 2012). For pure burger, 100 g specimen of minced dog, chicken and beef meats were added with 1.2 g finely chopped tomato, 0.2 g onion, 0.2 g egg, 1 g cumin seed, 0.25 g cayenne pepper than mixed well and each separate mixture was given to a burger shape. To simulate dog meat adulteration, chicken and beef burgers were prepared by spiking 10%, 1%, 0.1% and 0.01% of dog meat into chicken and beef meats, mixed and minced well. The raw burgers were autoclaved at 133 °C at 45 psi for 20 min according to European legislation (Commission, 2002). The raw burgers meat were grilled on both sides in an electrical oven at 220 °C for 15 min and placed with tomato, cucumber and buns to simulate ready to eat burger formulation. Thus prepared all burgers were kept at -20 ^{0}C and were blended well into a homogenous mixture prior to DNA extraction.

6.2.7 RFLP Analysis

For RFLP analysis, PCR products were digested with *Alu*I restriction enzyme in a 30 µl reaction mixture containing 10 µl of PCR product, 1 µl of enzyme (1 FDU), 17 µl of distilled water and 2 µl of 10x digestion buffer supplied with the enzyme (New England Biolab, USA). Firstly, the mixtures were gently mixed and spin downed and then incubated at 37 °C in a water thermostat for 15 min to performed digestion. After digestion, the enzyme was inactivated by heating the mixture at 65 °C for 5 min. Finally, RFLP analysis was performed by running 1µl of the restriction digested products of each sample in Experion™ lab-on-a-chip bioanalyzer system using 1K DNA analysis kit (Bio-Rad Laboratories, USA).

6.3 Results and Discussion

6.3.1 Canine species specificity

Species-specific PCR assay is a commonly used technique for species authentication in raw and processed foods (Mane, Mendiratta, & Tiwari, 2009). Presence of even single mismatch in the primer binding site may reduce the efficiency of PCR assay or may lead to the failure in PCR amplification (Wu, Hong, & Liu, 2009). Therefore, calculation of oligonucleotide mismatch is one of the key factors to be considered while designing species-specific primers. Designing primers with perfect matching with the specific target and multiple-mismatches with non-target species would definitely increase the specificity of the primer, decreasing chances of non-target amplification.

Blast analysis against non-redundant nucleotide sequences using NCBI data based revealed that the primers were 100% specific for canine cytb gene. Sequence alignment test against cytb/cob gene sequences of total 20 potential animal and plant species using clustalW multiple sequence alignment program demonstrated 100% similarity with canine cytb gene and multiple mismatches with those from other species (Fig 1.c). Pair wise distances for 100 bp canine specific sites computed by Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004) ranged from 0.26 to 2.15. The lowest distance was between dog and sheep (0.26) and highest was between dog and tomato (2.15). Construction of dendogram also revealed maximum sequence similarity of dog with sheep (Figure 6.2. a). The number of oligonucleotide mismatches of all other species except dog in the primer binding sites was 5-11 (Figure 6.2 b). Thus a 3D plot using pair wise distance and number of primer mismatches reflected clear discrimination of the canine primers from all other species including sheep (Figure 6.2 c). A real-PCR analysis was done against the total DNA of 7 commonly used meat

animals (chicken, quail, sheep, goat, beef, buffalo, pig), 1 plant (cucumber) and 1 fish (tilapia) species those were demonstrated minimum number of oligonucleotide mismatches in theoretical analysis (Figure 6.2. c). The assay amplified only 100 bp canine cytb gene target at an optimized annealing temperature of 58 ^{0}C, and a primer concentration of 0.4µM (Figure 6.3). An universal 141 bp fragment of eukaryotic 18S rRNA gene target amplified from all species (Figure 6.3), demonstrating both the specificity of the canine primers and eminence of good quality DNA from all species used in this study (Rojas et al., 2010).

Previously proposed canine specific PCR-RFLP assay was based-on mitochondrial cytb gene (Abdel-Rahman et al., 2009) with larger amplicon size (808 bp; ≥150 bp) which may breaks down under food processing condition, causing failured of the PCR assay. We used here shorter DNA target (100 bp) which was stable under various food processing treatments as we had tested and described discussed in theprevious chapters in relevant sections (Chapter 3-5).

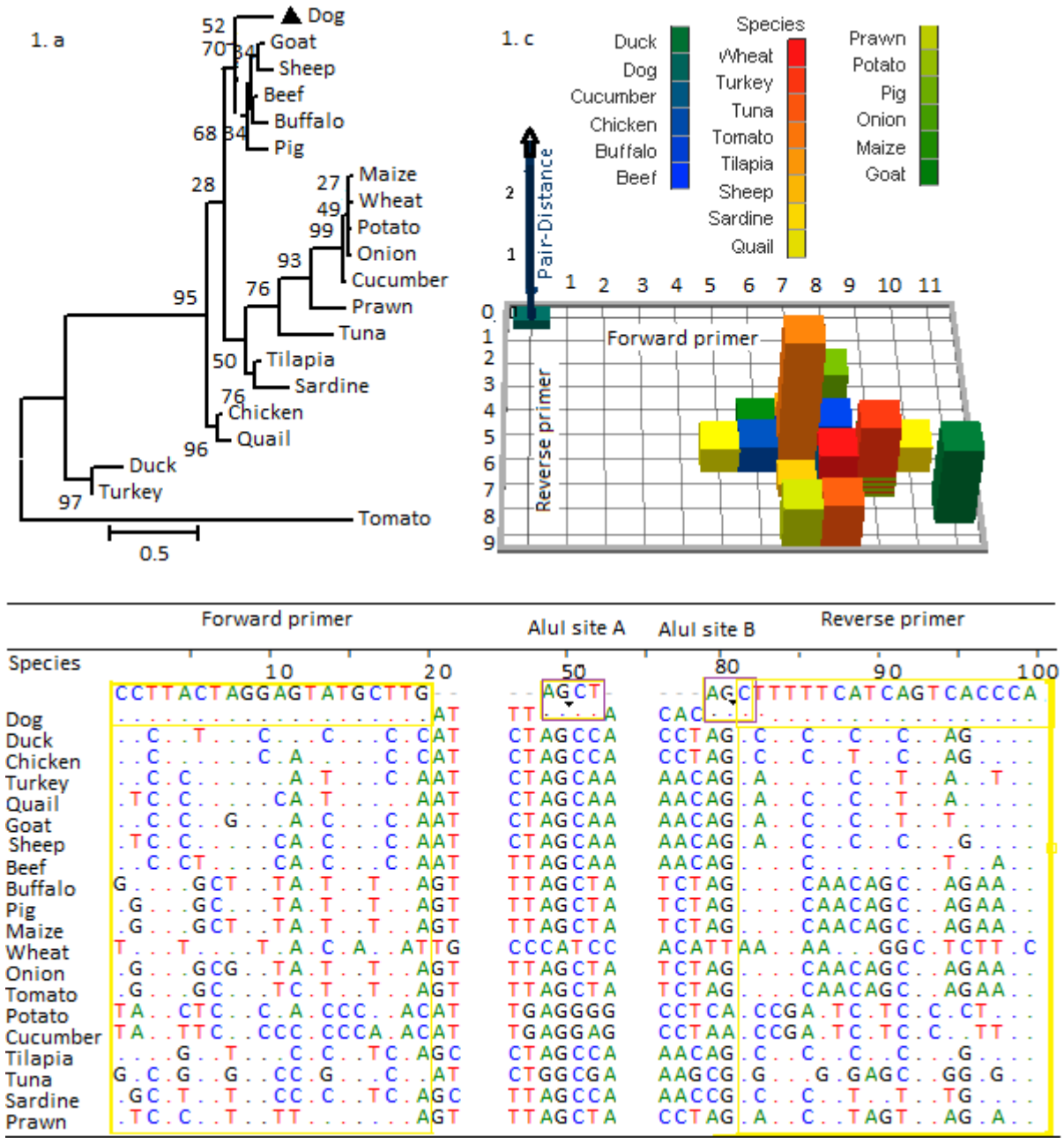

Figure 6.2 In-silico analysis of the canine specific primers under burger formulation. a) Dendogram built from the 100 bp regions of cytb/cob-gene sequences of dog and other 19 animals, plant and fish species using Neighborhood-Joining method. b) Mismatch bases of studied species with canine specific primers and *Alu*I restriction sites. c) Analysis of the primer mismatch and pair wise distance using 3D plot.

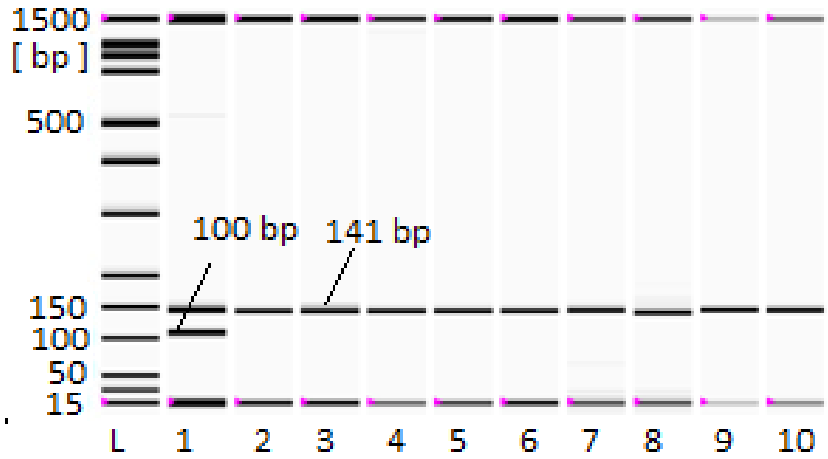

Figure 6.3 Canine-specificity analyses using lab-on-a-chip gel, showing 100 bp PCR product from dog and 141 bp eukaryotic endogenous control from all species DNA template. Lane L: DNA ladder; Lane 1-10: dog, chicken, quail, sheep, goat, beef, buffalo, pig, cucumber and tilapia, respectively.

6.3.2 Assay sensitivity

The 100 bp canine specific site was tested to determine the sensitivity of the assay using 10-fold serial dilutions (100, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 ng) of the extracted canine DNA with distilled water. Clear gel-bands (Figure 6.4) and electropherograms (data supplementary table) for canine-specific PCR products were observed with as low as 0.0001 ng of canine DNA extracted from raw meat. Thus the detection limit (LOD) of the assay was determined to be 0.0001 ng canine DNA which was equivalent to an automated and expensive real-time PCR (Yusop, Mustafa, Che Man, Omar, & Mokhtar, 2012).

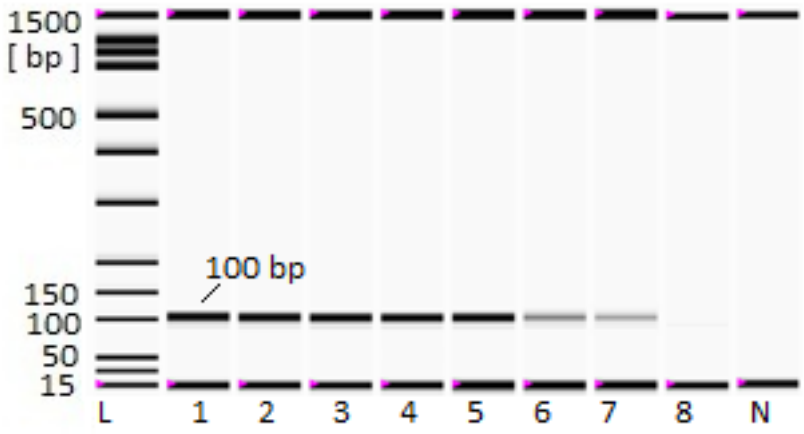

Figure 6.4 Sensitivity analyses showing a detection limit of 0.0001 ng (Lane 7) total DNA extracted from pure canine meats under raw states. Lane L: DNA ladder; Lane 1-7: 100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001 ng, 0.0001 ng and 0.00001 ng canine DNA. Lane N: Negative control.

The higher sensitivity of this PCR assay might be due to shorter amplicon-length (100 bp). Rodríguez, García, González, Hernández, & Martín (2005) obtained 0.01 ng sensitivity for 411 bp fragment of porcine mt-12S rRNA gene. On the other hand, using a shorter amplicon (119 bp) and molecular beacon real-time PCR (Yusop et al., 2012) recognized 0.0001 ng LOD for porcine mt-cytb gene. (Ali, Hashim, Mustafa, et al., 2012) also documented 0.0001 ng LOD for a 109 bp site of porcine cytb gene using convention PCR coupled to microfluidic bioanalyzer chip. Thus the 0.0001ng LOD for a 100 bp target of canine cytb gene using lab-on a chip technique for PCR end point detection was acceptable and the assay would be useful to determine a trace amount of canine DNA in processed foods.

6.3. 3 Binary admix analysis

To stimulate commonly practiced meat adulteration, we prepared two sets of admixtures following (Ali, Hashim, Mustafa, et al., 2012). Set 1 was dog-chicken and set 2 was dog-beef binary admixtures containing various percentages (10-0.01%) of spiked dog meat. We subjected sets 1 and 2 to extensive autoclaving (2.5 h) to test the target stability under extensive heat and pressure treatments. We obtained canine specific (100 bp) PCR product from all chicken and beef admixed containing as low as 0.01 % (w/w) (20 ng total and 0.002 ng canine DNA) of spiked dog meat (Figure 29. a). An endogenous 141 bp eukaryotic targets were amplified from all admixed, reflecting good quality DNA in all admixtures (Figure 6.5 a).

For canine-species detection under binary matrix (Martín et al., 2007) documented a species-specific PCR assay targeting 101 bp fragment of 12S rRNA gene in meat-oats binary admixtures under normal autoclaving condition. The sensitivity of that assay for dog meat detection was 0.1% (w/w) (about 0.125 ng DNA). The assay was less sensitive than the present one in terms of the amount of template DNA used (125 ng vs. 20 ng) and the limit of detection (0.1% vs 0.01%). For dog meat detection, previously we have documented cytb based PCR assay using agarose gel end point detection technique with a sensitivity of 0.1% to 0.2% (w/w) (0.02-0.04 ng DNA) (Chapter 3-5). Compare to this, the present assay using CE based automated end point detection scheme provided a higher sensitivity of 0.01% (w/w) (0.002 ng canine DNA) using similar admixtures of dog meat with chicken and beef.

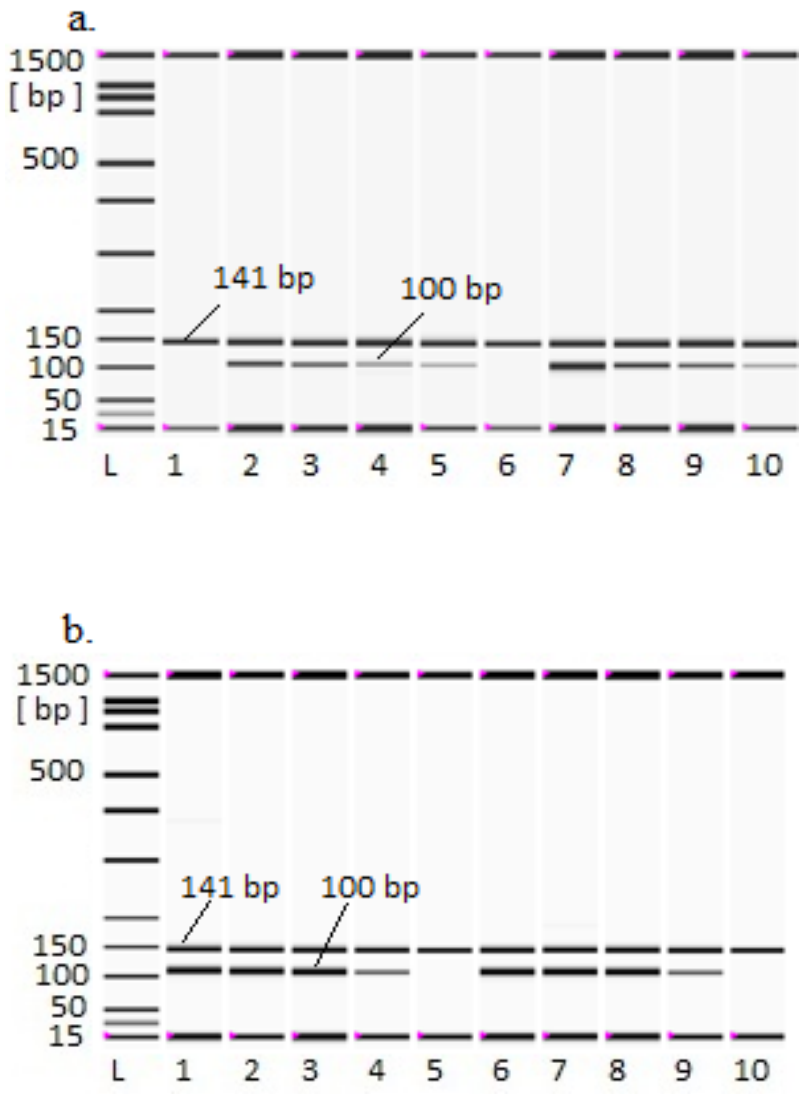

Figure 6.5 Specificity and sensitivity analysis of the canine specific assay using lab-on-a-chip gel image, showing 10-0.01% (w/w) meat or (20-0.002 ng canine DNA) of canine meat adulterated chicken and beef binary admixtures a) and burger b). Shown are in a): Lanes 2-5: 10%, 1%, 0.1% and 0.01% dog meat admixture with chicken; Lanes 7-10: 10%, 1%, 0.1% and 0.01% dog meat admixture with beef. Lane 1 and 6: 100% chicken and 100% beef. In b: Lane 1-4: 10%, 1%, 0.1% and 0.01% dog meat spiked chicken burger and Lane 6-9: 10%, 1%, 0.1% and 0.01% dog meat spiked beef burger. Lane 5 and 10: 100% chicken and 100% beef burgers. Lane L: DNA ladder. PCR products shown are 141 bp eukaryotic endogenous control and 100 bp for canine specific cytb site.

6.3.4 Model burger analysis

Food adulteration is often performed in minced meat products such as burgers (Ali, Hashim, Dhahi, et al., 2012), sauces and meatballs. Among these items, burgers, especially beef and chicken burgers, are very popular in all continents of the world (Ali, Hashim, Dhahi, et al., 2012). Recent documentation on rat and horse meat scandal in China and Europe (Ali, Razzak, & Hamid, 2014) has further prompted us to investigate whether canine meat is being substituted in popular burger formulations. Therefore, we tested the performance of the PCR assay using lab-on-a-chip end point detection scheme under complex background of minced meat products in pure as well as in deliberately contaminated format by spiking different percentages of dog meat (0.01% to 10%) with chicken and beef burger. We found that the assay can detect as low as 0.01% of canine meat contamination both in chicken and beef burgers (Figure 6.5 b).

Previously, Ali, Hashim, Mustafa, et al., (2012) reported a LOD 0.01% for ternary admixtures composed of pork, chicken and wheat using microfluidic detection method for a 109 bp site of porcine cytb gene. For burger analysis using the same target (109 bp) and real time PCR assay Ali, Hashim, Dhahi, et al., (2012) obtained 0.01% LOD. Recently, 0.1% (w/w) and 0.2% (w/w) LOD have been documented by us for canine species detection in frankfurter, kabsa, curry (Chapter 3, 5) and meatball (Chapter 4) formulations, respectively. However, these results were based on conventional PCR and agarose-gel electrophoresis for PCR end point detection by using short target of canine cytb gene. Thus a 0.01% LOD for canine specific target burger formulation using a highly sensitive microfluidic lab-on-a-chip detection technique and shorter (100 bp) target was an expected and logical outcome.

6.3.5 Burger authentication by RFLP

RFLP sites in 100 bp target of canine cytb gene was searched using NEB cutter version 2.0 and two cut sites (Figure 1. b) for *Alu*I restriction enzyme were obtained with three fragments (19-, 30- and 51-bp). The *Alu*I digested PCR products of raw and autoclaved meats along with raw, autoclaved and ready to eat dog burgers were separated by lab-on-a-chip microfluidic separation technology, and two clear fragments of 30 and 51-bp were visualized both in gel-image (Figure 6.6 a), and electropherograms (Figure 6.6 b). The gel-bands and electropherograms for the19 bp fragment were merged with the 15 bp lower marker and appeared as a thicker band for the lowest ladder (Figure 6.6 a, b) since the technique have the limitation to resolute ≤5 bp difference in fragment length (Garino, et al., 2014). The molecular size statistics of the 100 bp canine specific-site from raw and autoclaved meat along with raw, autoclaved and ready to eat model dog burgers are given in Table 6.1.

Conventional PCR is an essential tool for routine analysis in bioanalytical, clinical, and research laboratories. But certain “difficult-to control” features of the amplification process of the PCR has yet to be optimized (Ali, Hashim, Mustafa, et al., 2012; Yang, Kim, Byun, & Park, 2005). These features generally may derive from the artifacts of simultaneous amplification of small contaminants at high magnitudes and non-specific target amplification (Doosti, Ghasemi Dehkordi, & Rahimi, 2011; Yang et al., 2005). PCR product architecture with restriction site can unambiguously differentiate the original target. Thus both in-silico digestion and real RFLP analysis of 100 bp PCR product digested by *Alu*I restriction enzyme yielded three canine specific fragments and eliminated the probability of misleading results by non-specific contaminant amplification. Quantitative real-time PCR assay with automated detection technique by real-time monitoring of the amplification cycle is theoretically interesting but it often produces artifacts and incurs excessive cost (Bustin et al., 2009) in terms of instrument,

reagents and probes. On the other hand, PCR-RFLP analysis produces enhanced specificity of the PCR assay with a unique restriction pattern from each species of animals (Aida, Che Man, Wong, Raha, & Son, 2005; Ali, Hashim, Mustafa, et al., 2012; Doosti et al., 2011; Sait et al., 2011). Previously, (Abdulmawjood, Schönenbrücher, & Bülte, 2003) documented a PCR-RFLP assay of 808-bp amplicon length for canine species detection. Such a large-size amplicon had the possibility of breaking down during the processing treatments, causing PCR failure while testing the commercial foods. Thus, the advantage of our PCR-RFLP assay is easily understandable in terms of sensitivity, amplicon-length and stability.

Table 6.1 Molecular sizing statistics of lab-on-a chip based canine specific PCR-RFLP assay.

Size (bp)	Raw dog meat with *Alu*I digestion		Autoclaved dog meat with *Alu*I digestion		Raw dog burger with *Alu*I digestion		Autoclaved dog burger with *Alu*I digestion		Ready to eat dog burger with *Alu*I digestion	
	Before	After	Before	After	Before	After	Before	After	Before	After
100	101±.5		100±1.2		100±0.8	-	100±0.5		100±0.8	
51		52±.5		51±.5		51±.0		51±.5		51±0.5
30		30±.5		29±.8		30±.5		30±.8		29±.9
19	-	-	-	-		-		-		-

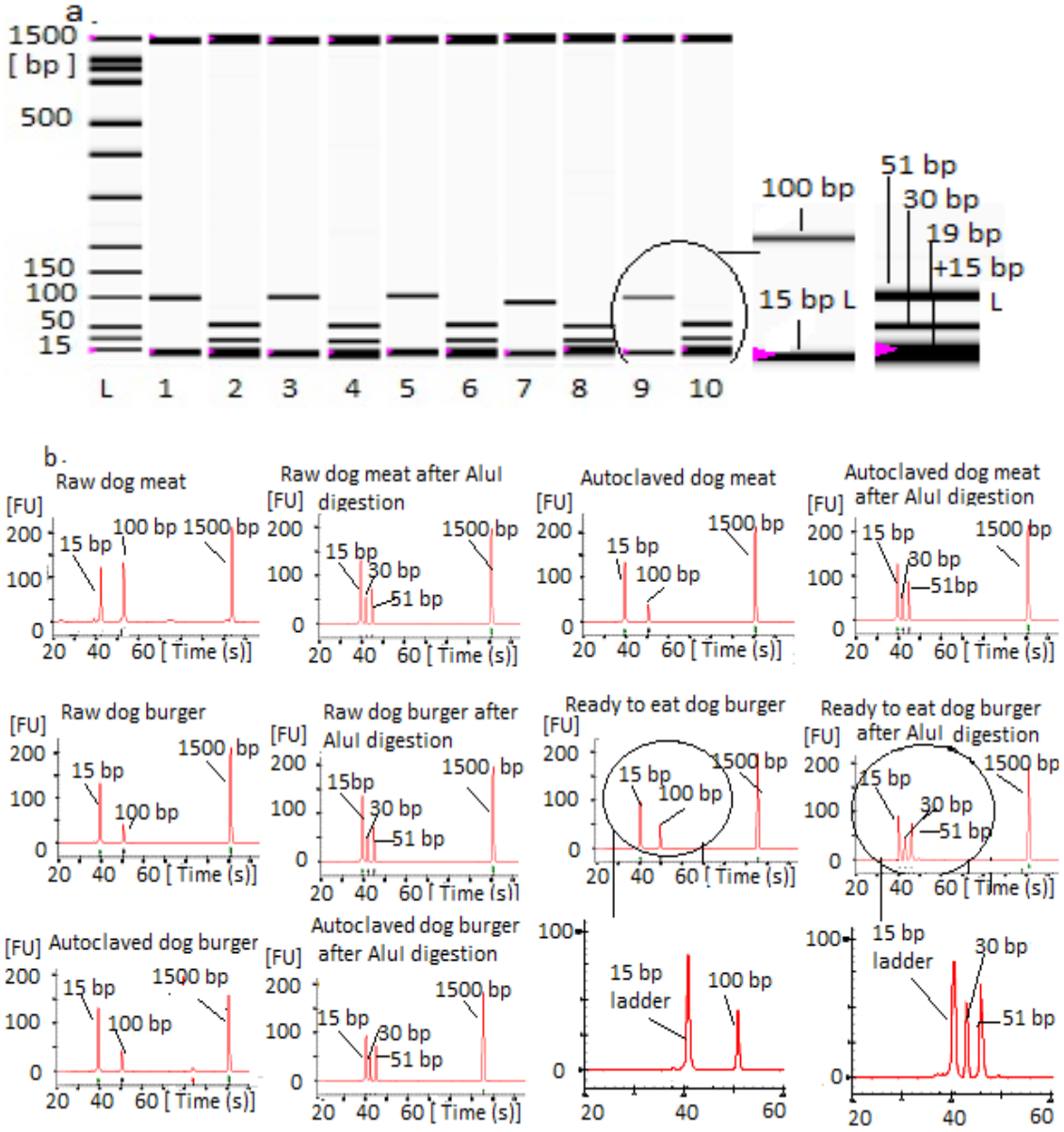

Figure 6.6 PCR-RFLP analyses, showing 100 bp PCR products and *Alu*I restriction digestion products obtained from raw, autoclaved and ready to eat model dog burger. a. In gel image Lane L: Ladder; Lane 1, 3, 5, 7, 9 before restriction digestion and Lane 2, 4, 6, 8, 10 after restriction digestion of PCR product obtained from raw, autoclaved and ready to eat model dog burger, respectively. b. Corresponding electropherograms are demonstrated by respective labels.

6.3.7. Commercial burger analysis

In food industry, replacement of costly meats by cheap ones is quite common for the realization of extra profit. Therefore, we screened here commercial burger samples using 0.01% dog meat spiked dummy burgers as a positive control. A Total eight of different "Halal" branded chicken (A-D) and beef burgers (A´-D´) purchased from different Malaysian outlets were tested. While canine PCR-product was obtained from all positive controls, no commercial burgers collected from different outlets were found to be positive for dog meat (Figure 6.7, Table 6.2), reflecting the absence of dog-meat adulteration in burger formulations in Malaysia. Amplification of endogenous eukaryotic control, reflected for good quality DNA in all PCR products. The findings are acceptable in Malaysian perspectives since the country is committed to develop Hala-hub industry and strictly monitoring the Halal status of foods.

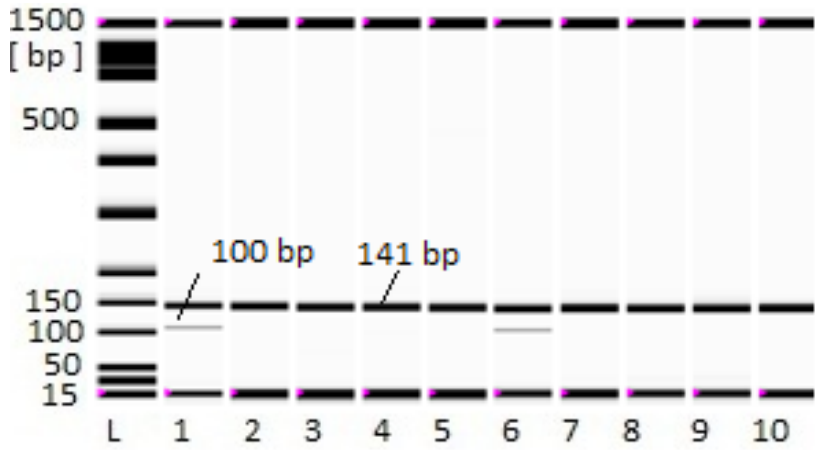

Figure 6.7 Commercial chicken and beef burger analysis using lab-on a chip based assay. Amplified PCR products are canine specific 100 bp site from 0.01% dog meat spiked chicken (lane 1) and beef burger (lane 6) and 141 bp endogenous control (Lanes 1-10). Lanes 2-5: commercial chicken and Lane 7-10 beef burgers. Lane L: DNA Ladder.

Table 6.2 Analysis result of commercial chicken and beef burgers collected from different outlets of Malaysia.

Chicken burger	No of sample	≥0.01% detection	Beef burger	No of sample	≥0.01% detection	Detection probability
A	3	0/3	A'	3	0/3	100%
B	3	0/3	B'	3	0/3	100%
C	3	0/3	C'	3	0/3	100%
D	3	0/3	D'	3	0/3	100%
Positive control	3	3/3	Positive control	3	3/3	100%

The usual halal burger formulation composed of chopped meat of chicken, beef, lamb or fish along with starch, seasonings and salt. Replacement of dog meat in the commercial burger may add an extra profit. Species specific PCR is one of the widely used methods for meat species authentication. To detect fraudulent admixing of canine meat a number of species specific assays have been reported (Abdel-Rahman, El-Saadani, Ashry, & Haggag, 2009; Abdulmawjood et al., 2003; Gao et al., 2004; Martín et al., 2007). However, none of those assays were tested in commercial burger background, while reports have been made about the PCR inhibition possibilities in commercial or process food due to numerous factors (Bottero, Civera, Anastasio, Turi, & Rosati, 2002; Di Pinto, Forte, Conversano, & Tantillo, 2005). In the previous chapters 3, 4 and 5; we have described the canine specific PCR assay for commercial frankfurters, different cuisines and meatballs formulation with a positive control of 0.1% and 0.2% (w/w) deliberately spiked dog meat using agarose gel end point detection. For the first time, we documented here a PCR-RFLP assay with lab-on-chip microfluidic-based enhanced detection platform and obtained extremely high sensitivity 0.01% (w/w).

6.4 Conclusion

Analytical method described here utilized both canine specific PCR and RFLP analysis coupled with enhanced microfluidic-based lab-on-a-chip detection platform to determine dog meat adulteration in raw, autoclaved as well as ready to eat processed burgers. The technique combined the use of canine specific primers together with eukaryotic endogenous control to probe the quality of DNAs used in each experiments. The efficiency and sensitivity of the assay were tested in raw, admixed and commercial burger backgrounds and high sensitivity (0.0001ng DNA for raw pure meats and 0.01% (w/w or 0.002 ng DNA for binary admixtures and beef and chicken burgers) were obtained. Additionally, the canine specific PCR product was confirmed by RFLP analysis, eliminating confusion and doubt about the authentic target. The assay was validated in burger formulation. The screening of halal-branded commercial burgers did not show any positive results for canine meat in Malaysian outlets.

CHAPTER 7
TAQMAN PROBE REAL TIME POLYMERASE CHAIN REACTION ASSAY FOR THE QUANTIFICATION OF CANINE DNA IN CHICKEN NUGGET

7.1 Introduction

7.1.1 Chicken nuggets

A chicken nugget is a breaded or buttered chicken product. It is made from chicken meat slurry or chicken breast cut in to small shape and deep fried or baked. It is sold as breaded or raw; as gluten-free or organic; fried or grill-marked. It is a famous kid's menu with a variety of size and shape like stars, alphabets, or dinosaurs. The current spongy, small shaped chicken nuggets was reviled by Jamie Oliver who recreated the Michael Pollan's ones by whizzing chicken meat slurry in a food processor and infiltrating it through a sieve. However, the first chicken nugget was invented in 1950 by Robert C. Baker from Cornell University (McKenna, 2012). Later, in 1963 he published an unpatented academic study on chicken nuggets structure which can be formed in any shape and proposed a model for frozen, breaded "chicken stick". The current McNuggets recipe from chicken by McDonald's was created by Tyson Foods in 1979 on commission from McDonald's, and the product was first sold at the beginning of 1980. Now a day, nuggets are available in most of the fast-food chain and can be found in most of the supermarket's freezer. Last year in only in USA, total sale of nuggets were 29.9 million dollars beside other meat snacks (Figure 7.1).

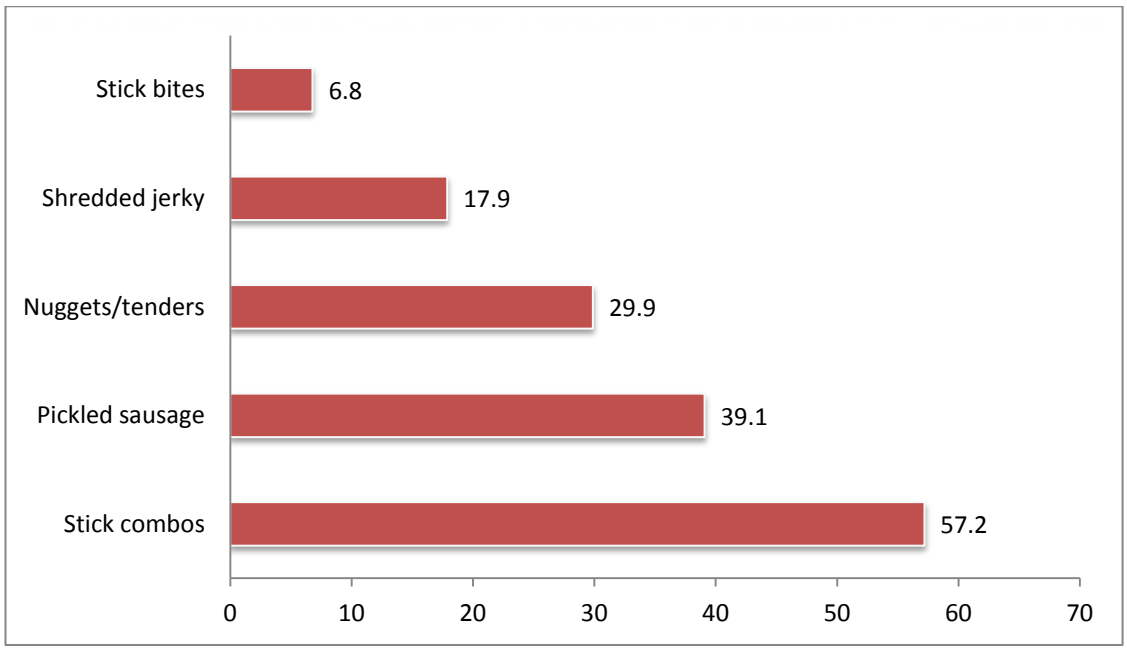

Figure 7.1 Sales of meat snacks in U.S. convenience stores in 2013, by type (in million U.S. dollars) (Information Resources, 2013).

7.1.2 Current chicken nuggets scandal

The horse-meat scandal driven over the Europe in the year (2013) was also associated with chicken nuggets. The health authorities of Greek had recalled certain meat products including chicken nuggets due to the presence of horse meat. The Hellenic Food Safety Authority (EFET) determines the chicken nuggets adulteration with horse meat using laboratory analysis of the DNA. The result confirmed the presence of 10 to 25 % horsemeat in chicken nuggets and the authority recalled the country's products chicken nuggets in Greece (Embiricos, 2013). The world giant fast food chain McDonald's was hanged the sales of chicken nuggets in Hong Kong in July 2014, due to the recent meat scandal in China (Figure 7.2). The Chinese company Shanghai Husi Food was supplying long expired meat. Thus McDonald's Holdings Co (Japan) halted the purchase of chicken products from China and changes the business to Thailand (Yates & Geoghegan, 2014). A study on chicken nuggets from two American fast food company revealed that it was containing 50 percent of chicken meat but the

rest was composed of bird's internal organ, skin, fat, nerves, blood vessels, cartilage and bone fragments (Doyle, 2013).

Figure 7.2 "Temporary" unavailability of chicken nuggets in a McDonald's restaurant in Hong Kong (Yates & Geoghegan, 2014).

7.1.3. Prospect of current canine specific RT-PCR assay

Meat is a major source of protein in human daily diet with its high demand (Soares, Amaral, Oliveira, & Mafra, 2014). Food adulteration with materials derived from other species with greater availability and/or lower cost in the meat industry have become a worldwide problem (Drummond et al., 2012). Recent meat product adulterations scandal in Europe has given a great strike in the consumer's belief regarding the composition of the food product they eat. Hence, identification of animal species of commercial food has become a vital part in value chain analysis to secure consumer trust and to assure the correct labelling of the origin of the species of food product (Rojas et al., 2011). Furthermore, zoonotic diseases, such as consumption of

meat from BSE-infected animals caused death of about 200 people worldwide, including Canada and United States. Hence species authentication is a vital part for the risk assessment of animal derived materials for human health (Corona, Lleonard, Carpio, Uffo, & Martínez, 2013). For identification of animal species in food products, analytical tools are necessary while food processing caused drastic changes in raw materials (Woolfe & Primrose, 2004). In the previous chapters (Chapter 3-5), we have described qualitative detection of the canine species in raw or processed food products. Although real time PCR is a highly sensitive method and allow quantitative detection platform (Chapter 2, Section 2.13.6) but there is no quantitative assay for dog meat detection has been reported yet. Therefore, the current study aimed to develop a highly sensitive and quantitative assay platform for canine DNA species detection for determining the food adulteration or halal authentication.

7.2 Materials and Methods

7.2.1 Collection of samples

The samples of eight commonly used meat providing species such as chicken, duck, turkey, goat, sheep, cattle, buffalo, pig and two common plant materials such as soybean and wheat were purchased from the various supermarkets located at Kuala Lumpur and Selangor in Malaysia. The collection of dog meat was done from Dewan Bandaraya Kuala Lumpur, Malaysia and Faculty of Veterinary Science, University Putra Malaysia For commercial nuggets analysis, chicken nuggets of three "halal' different brands were purchased from different supermarkets/store chain located in Kuala lumpur, Malaysia. All the samples were collected in triplicate in three different days and transported at ice chilled condition (4 ^{0}C) to prevent the DNA degradation. Samples were stored at -20 ^{0} C for future work and DNA extraction.

7.2.2 Chicken nuggets preparation

For preparation of nuggets, the muscle samples of chicken and dog were taken from skeletal muscle (95%), intestine (2.5%), liver (1%), heart (0.5%), kidney (0.5%) and 0.5% skin for representation of the typical tissue composition of the farm animal's meat. The samples were chilled overnight at 4 °C and deboned manually cut into small pieces and minced. For the simulation of dog meat contamination in nuggets, meat mixtures were prepared by spiking 0%, 0.01%, 0.1%, 1%, 10% and 100% (w/w) of dog meat in a 250 g portion of each spiking level with chicken. The meat admixtures minced twice using a 4 mm plate with meat mincer (Sin Huat Hin, Malaysia). To a 200 g portion of each meat mixture, 10 g soybean oil, 10 g textured soya protein, 10 g chilled water, 5 g refined wheat flour, 15 g finely chopped raw carrot, 10 g whole egg liquid, 5 g condiments (onion and garlic paste), 3 g spice mix, 1 g sugar, 2 g sodium

chloride, 25 mg sodium nitrite and 0.4 g tetrasodium pyrophosphate were added. By vigorous blending an emulsion of each admixture was prepared and steam cooked manually in stainless steel molds. The lid was tightly closed and the emulsions were steam-cooked at an internal temperature of 80–85 °C for 15 min. Small nuggets of uniform sizes were made after subsequent cooling of meat emulsions under room temperature.

7.2.3 Calibration and validation standard

For calibration set, chicken nuggets were spiked with 0.01, 0.1, 1.0, and 10.0% (w/w) of dog meat. A different set of nuggets were also prepared with similar composition for the validation of the prediction model.

7.2.4 Extraction of DNA

DNA was extracted from 25 mg of raw meat samples using NucleoSpin® Tissue extraction kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. For different plant species (100 mg) and spiked nugget samples (1 gm); DNA was extracted using appropriate CTAB method for plant and animal tissue as described in the chapter 3 sections 3.2.2. Subsequent purification was done by Promega Wizard™ DNA isolation kit (Promega Corporation, Madison, USA). The concentration and purity of the DNAs were determined using UV-vis spectrophotometer Biochrom Libra S70 (Biochrom Ltd., UK).

7.2.5 Primer and probe design

A pair of canine specific primers (CacytbF and CacytbR) targeting 100-bp fragment of canine cytb gene (Dog: JF489119.1) was designed using publicly available primer3Plus software (www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi). A

24-nt canine specific TaqMan probe (CacytbTqM) was designed by tagging 6-carboxyfluoresceine (6-FAM) and 3-Iowa black FQ (3-IABkFQ) at the 5′- and 3′-ends. For endogenous control, eukaryotic 18SrRNA-specific primers (Eu18SrRNAF and Eu18SrRNAR) and a TaqMan probe (Eu18SrRNATqM) described by Rojas et al. (2010) were used. All the primers and probes were purchased from IDT, USA and are shown in Table 7.1.

Table 7.1 Primers and probe sequences used for nugget analysis using RT-PCR

Name	Sequences (5'-3')
CancytbF	CCTTACTAGGAGTATGCTTG
CancytbR	TGGGTGACTGATGAAAAAG
CancytbTq	6-FAM/AAGTGGACT/ZEN/ TGCACTATACATCGGACACAGCCA /3IABkFQ
Eu18SrRNAF	GGTAGTGACGAAAAATAACAATACAGGAC
Eu18SrRNAR	ATACGCTATTGGAGCTGGAATTACC
Eu18SrRNATq	6-FAM/AAGTGGACT/ZEN/CATTCCAATTACAGGGCCT/3IABkFQ

7.2.6 Real-time PCR assay

Real-time PCR was performed in Eppendorf Mastercycler ep-realplex machine (Eppendorf, Germany) with 20µl reaction mixture consisting of 1× SsoFast probe supermix (Bio-Rad, USA), 200 nM of TaqMan probe, 300 nM of each primers and 20 ng of genomic DNA template. The dilutions were prepared using sterile deionized water. For PCR amplification, a two-step amplification program was optimized at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 58 °C for 20 s. Endogenous control and canine specific RT-PCR assays were performed in separate tubes. Each sample and endogenous control was run in triplicates on three different days by three independent analysts.

7.2.7 Construction of standard curve and target quantification

The quantification of canine DNA in different chicken nuggets was done by interpolating the normalized value the number of threshold cycles (Ct) at which the fluorescence exceeded the threshold with a standard curve. The standard curve was generated from Ct values against the known concentration of the DNA. For the calculation of Ct value of the canine-specific system (CtPS), the following equation was used as described by Rojas et al., (2010).

$$CtNPS = CtEp \text{ X } CtPS/CtEB \quad (1)$$

Where *CtNPS* represents the normalized Ct value of the sample with canine-specific PCR system, *CtEp* is the average Ct value of 20 ng canine DNA from nuggets formulation in endogenous PCR system, and *CtEB* is the Ct value of the specific nuggets sample with the endogenous PCR system. The validation experiment with chicken nuggets samples at 95% confidence level with various amount of spiked canine meat did not show any significant changes in the Ct values of endogenous system. Therefore, it was assumed that *CtEP = CtEB* and thus the simplified form of the above Equation 2 was derived.

$$CtNPS = CtSP \quad (2)$$

It demonstrated no significant statistical difference between the normalized Ct and the unprocessed Ct of the canine-specific system in chicken nuggets formulations.

7.2.8 Statistical analysis and validation

Real time data analysis and Anova test were performed by Minitab 14 software (Minitab, State College, Pensylvannia, USA) and Xlstat 2013 (Addinosfot, 2013). For mean analysis, Tukey's Honesty Significant Difference (HSD) method was used along with Best model selection with Adjusted R^2. This model can handle a number of variables varying from "Min variables" to "Max Variables".

7.3 Result and Discussion

7.3.1 Taqman- Real time PCR assay

This real-time PCR assay for canine DNA quantification targeted a short fragment of canine cytb gene (100 bp) using canine specific primers and TaqMan probe. A 141bp site of eukaryotic 18S rRNA gene was used as reference target for assay normalization (Rojas et al., 2011). The application of an endogenous reference gene in real time PCR assay aids in the quantification of accurate target and prevent false negative detection. It eliminates the effects of other affecting factors such as expired reagents and presence of nucleic acid inhibitors in reaction vessels (Rojas et al., 2011). For compromised samples such as processed foods, endogenous control is a must since disintegrated and low purity DNA extracts are often obtained from commercial and processed meat products. An endogenous system traces unamplified DNA by the species-specific detectors and supports in comparison of signals acquired from the species-specific and endogenous control (Soares, Amaral, Oliveira, & Mafra, 2013; Soares et al., 2014). Thus it reduces factual errors caused by the standards and the unknown samples (Rojas, et al., 2010). For the validation of proposed RT-PCR assay, we have followed the standard analytical procedure to define the amplification efficiency of the primers and probe. Thus, triplicate samples of each amplification target and two sets of canine meat spiked model nuggets were used to validate this current assay. The potentiality of the proposed assay was verified by successful amplification of the canine specific target with a high specificity and sensitivity.

For canine specificity, firstly the designed primers and Taqman probe were tested by in-silico using blast tool in NCBI and alignment analysis with ClustalW alignment tool against a total of 8 commonly used meat providing animals including dog and 2 plants species used in nugget formulation. Finally, the PCR was run at higher annealing temperature (58 ^{0}C) which prevents non-specific primer binding and cross-species detection (Ali, Hashim, Mustafa, & CheMan, 2012). To amplify PCR signals, the intra-molecular distance between the 5′-fluorophore (FAM) and 3′-quencher (Iowa black) was reduced by inserting a second quencher, ZEN probe, at the 10th position of the TaqMan probe as described elsewhere (Ali, Hashim, et al., 2012). The developed PCR system was tested and validated for the analysis of chicken nuggets which contain multiple ingredients of complex matrices. The 100 bp target of multi-copy mitochondrial cytb gene was selected since short-length DNA targets have proven stability and sensitivity even in highly degraded samples and under extreme food processing conditions.

7.3.2. Canine specificity

NCBI blast analysis demonstrated the primers and probe had 100% identical sequence similarity with canine cytb gene. Alignment of both primers and probe sequences using ClustalW sequence alignment tool showed multiple nucleotide mismatches (forward 4-8, reverse 3-11, probe 5-13) with all the tested meat species (Figure 7.3 a, b, c). The pair wise distance of the 100bp canine site including the primers and Taqman probe were compared with the retrieved cytb gene sequences of 8 common meat-providing animals including dog and apocytochrome (cob) gene sequences of 2 plant (stated in sample collection section) using Maximum Composite Likelihood method (Tamura, et al., 2011). The lowest distance was between dog and sheep (0.26) and the highest was between dog and soybean (0.73). Drawing a 3D plot

using mismatched nucleotides in the primers and probe binding site clearly discriminated dog from all other tested species including sheep (Figure 7.3a). The presence of even single mismatch in the primer or probe binding site may reduce specificity of the assay and may lead to PCR amplification failure. Thus in-silico analysis reflected no probability of primer or probe annealing with any non-target species and cross-species detection. Further, a dendogram with alignment results of the 100-bp canine site with other species well separated the canine species from other meat and plant species commonly used in nugget formulation (Figure 7.3, b).

Finally, the cross-specificity of the canine-RT-PCR system was analyzed using 20 ng DNA from the muscle samples of 8 meat (dog, chicken, turkey, beef, buffalo, lamb, goat and pig) and 2 plants species (wheat and soyabean) and amplification signal was obtained only from canine target (Figure 7.4) in three repeated PCR run.The eminence of amplifiable DNA in all targets was demonstrated from the amplified endogenous control using universal eukaryotic primers and probe (Rojas et al., 2010). Ct values obtained from 9 replicates of canine DNA on 3 different days using the canine RT-PCR system were ranged from 16.13±.14 to 16.25±.23 (Table 7.2). The average Ct values of the animals and plants endogenous systems were between 18.26±0.14 and 32.82±.87 (Table 7.2). Thus, the endogenous control produced different spectrum with two major spectrums of Ct values for the animal and plant species used in this study (Figure 7.4) This TaqMan probe RT-PCR system effectively detected canine targets since both the primers and probe were dissimilar with other species and identical only with canine species (Rojas et al., 2011).

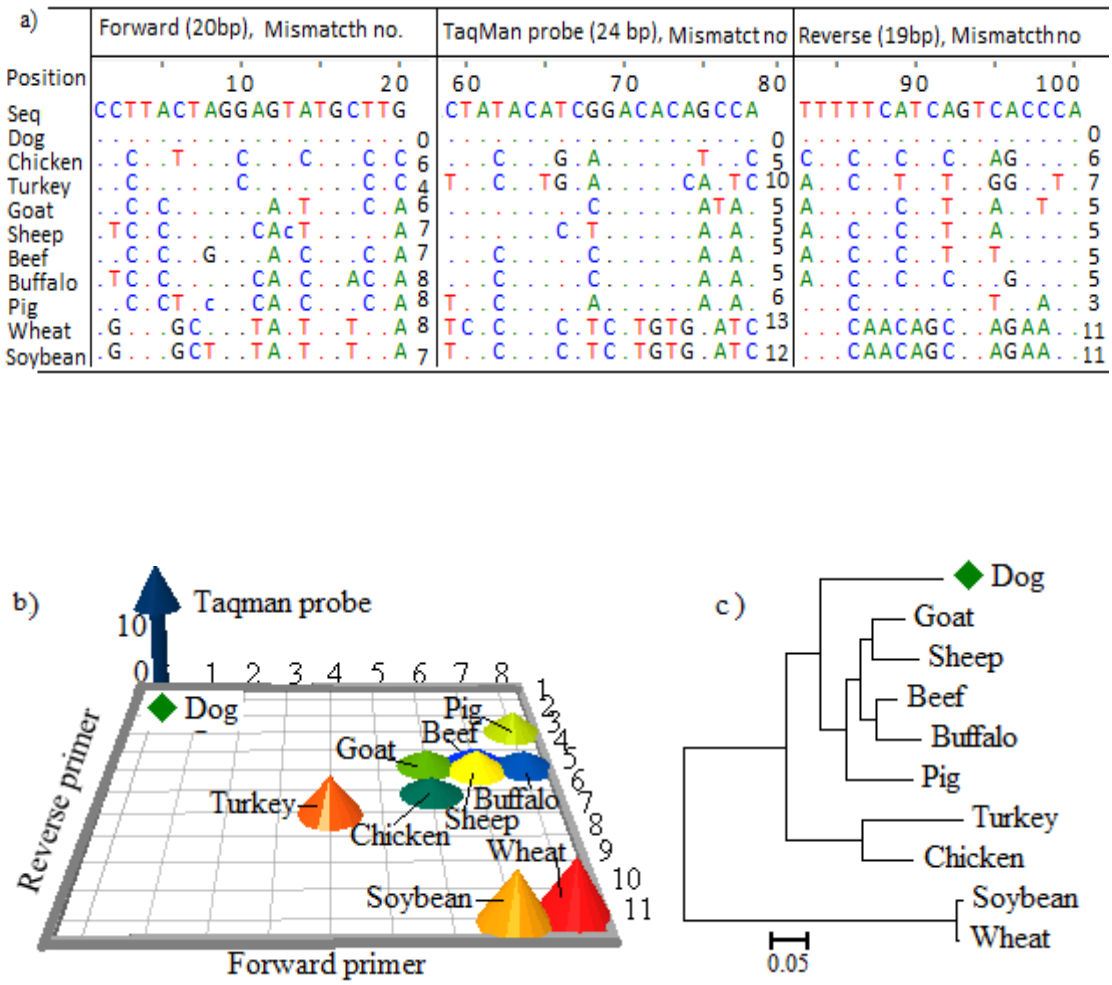

Figure 7.3 Insilco analysis of canine specific primers and probes along with 100bp canine specific site. Shown are in (a) the position of the primers and probes along with the mismatch count; in (b) 3D plot showing a clear discrimination of canine species using oligonucleotide mismatches in the primers and probe binding site and in (c) dendogram built by 100 bp canine specific site showing the separation of canine species from other common meat providing animal and plant species.

Table 7.2. Number of fluorescent quantification cycles from different species DNA.

Species	Mean Ct Canine specific PCR system			Mean Ct Endogenous control			No of positive replicate	
	Day1	Day2	Day3	Day1	Day2	Day3	Canine system	Endogenous control
Dog	16.25±.23[a]	16.2±.12[a]	16.13±.14[a]	19.41±.89d[e]	19.52±.10e	19.47± .19cde	3/3	3/3
Chicken	40[a]	40[a]	40[a]	19.81±.31[e]	19.41±.12e	19.51± .09cde	3/0	3/3
Turkey	40[a]	40[a]	40[a]	20.81±.23[cd]	20.27±.22de	20.93±.50c	3/0	3/3
Goat	40[a]	40[a]	40[a]	18.7±.17f	18.45±.18f	18.26±.10e	3/0	3/3
Sheep	40[a]	40[a]	40[a]	21.51±.19c	21.78±.23cd	20.56±.33cd	3/0	3/3
Beef	40[a]	40[a]	40[a]	18.61±.17f	18.43±.22f	19.21±.1.4de	3/0	3/3
Buffalo	40[a]	40[a]	40[a]	21.50±.28c	21.28±.09c	21.10±.20c	3/0	3/3
Pig	40[a]	40[a]	40[a]	20.77±.27cd	20.31±.23de	20.41±.23cd	3/0	3/3
Wheat	40[a]	40[a]	40[a]	29.98±.06b	29.48±.06b	29.93±.18b	3/0	3/3
Soybean	40[a]	40[a]	40[a]	32.82±.87a	32.32±.87a	32.36±.74a	3/3	3/3

Means with the same letter within the same column are not significantly different at 5% probability level.

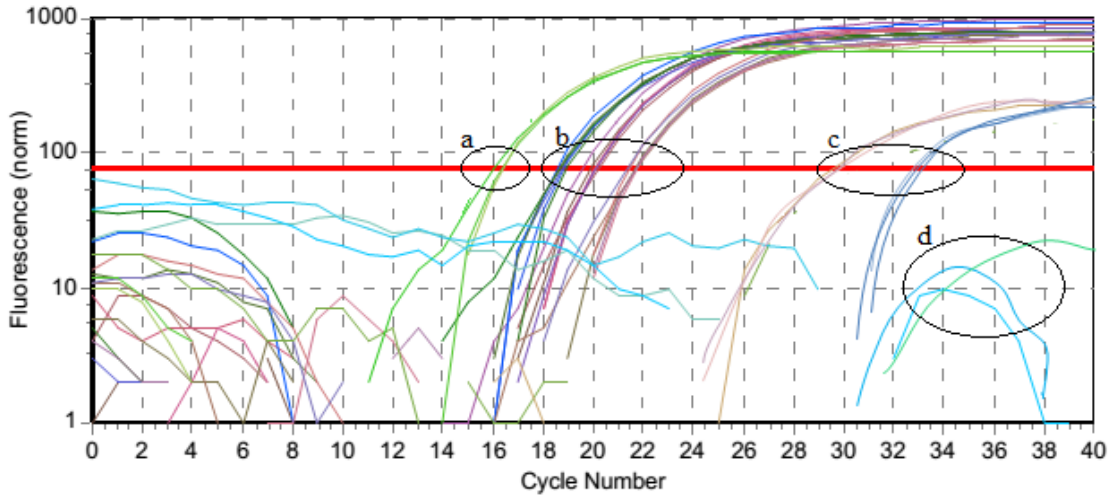

Figure 7.4 Fluorescent spectrum of PCR products amplified from canine specific (a and d) and endogenous (b animals and c plant) PCR systems. Each letter represents a total of three replicates from different extractions on one single day.

7.3.3 Efficiency and detection limit

To test the efficiency and detection limit of the canine RT-PCR assay, DNA samples from 100%, 10%, 1%, 0.1% and 0.01% of dog meat spiked chicken nuggets were analysed. Twenty ng DNA from each dog meat spiked nuggets was used to amplify both the canine target and eukaryotic control. In real time PCR assay, DNA quantification is performed from the detected fluorescence signals against the number of cycles on a logarithmic scale where a threshold of detection was slightly above the background noise. The number of threshold cycles (Ct) at which the fluorescence exceeded the threshold for different percentages of dog meat spiked nuggets were between 16.34±0.28 (100%) and 29.22±0.18 (0.01%). The mean Ct values of the eukaryotic endogenous control for different canine meat spiked nugget samples ranged from 19.79±0.13 to 20.51 ±0.36. The analysis of significance of the endogenous control Ct values at $P \leq 0.05$ using ANOVA test revealed no significant difference of endogenous Ct values for different percentage of canine and chicken. This might be due

to the sequence resemblance of 18S rRNA of dog (AY623831.1) and chicken (DQ018752.1), which allowed the similar target site for an absolute quantification even from different level of dog meat spiked chicken nuggets. We compared these sequences by alignment and indeed found 100% similarities between chicken and canine species.

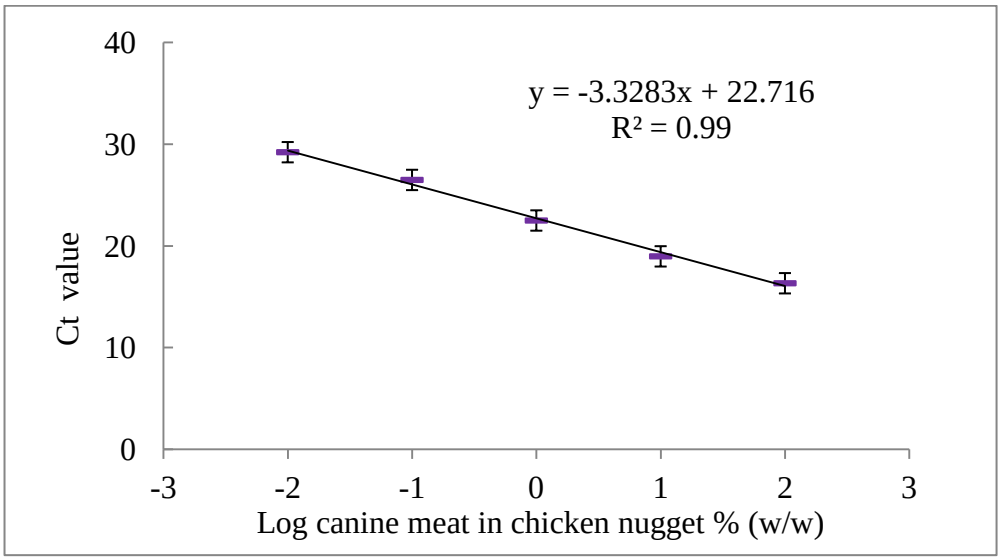

Figure 7.5 Normalized calibration curve obtained from different percentage (100% - 0.01%) of canine meat spiked chicken nuggets.

For the construction of a standard curve using real time PCR assay, Ct values were normalized (Rojas et al., 2010). Ct values obtained from a total of 45 replicates of chicken nuggets (three on three different days for five different samples) with different levels (0.01–100%) of dog meat contamination were analyzed at 95% confidence level. No significant differences in endogenous control eliminated the normalization of Ct value for the development of standard curve for the canine specific PCR system (Rodriguez et al., 2005; Rojas et al., 2010). Therefore, the logarithmic value of canine DNA in each formulation was plotted against the raw Ct values obtained from nine replicates of each nugget formulation of the canine-specific system (Figure 7.5). A good linear regression equation with a high correlation coefficient (R^2=0.99) and slope of -3.328 was found (Figure 7.5).

For the calculation of the PCR efficiency (E) in nugget formulation, the previously described method, E= [$10^{(-1/slope)}$ –1] was used (Fajardo et al., 2008; Yusop et al., 2011). A PCR efficiency of 99.7% from the present assay was obtained by using the current canine specific primer and probe. The previously described RT-PCR assay efficiency was varied from 90% to 110% (Ali et al., 2013) along with a high linearity (R^2 99). However, Rodriguez et al., (2005) obtained 64.8% and 68.9% efficiency in raw and autoclaved pork–beef binary admixture. The detection limit of the assay was 0.1% porcine DNA in pork–beef binary mixtures with a longer amplicon (411 bp) target using 12S rRNA porcine specific gene. Subsequently, using a comparatively smaller amplicon (119 bp) target and molecular beacon probe Yusop et al., (2012) obtained 96% efficiency. Recently, Ali et al., (2013) using Taqman Probe based RT-PCR assay reported an efficiency of 93.8% in deliberately spiked more complex form of meat adulteration in pork burger. Hence, the higher efficiency (99.7%) of the present assay could be conferred to the short length (100 bp) DNA target, multi copy mitochondrial cytb gene target, high linearity (R^2 0.99) and better DNA quality.

To obtain the real time PCR efficiency and detection limit, standard curve may be developed from ten-fold serial dilutions of pure (Yusop, et al., 2012)) samples or by using DNAs from binary species background composed of admixture of two different species (Rodríguez, García, González, Hernández, & Martín, 2005). However, ready to eat or processed foods are composed of more complex background with spices and food additives (Tanabe et al., 2007). Thus development of standard curve from pure or binary meat format background have the drawback of accuracy in quantification of the targets species for the commercial or ready to eat food products (Rojas et al., 2010). To overcome this limitation, we have prepared typical model commercial nuggets by spiking various percentages of dog meat. For better accuracy, different percentage of deboned meats (95% skeletal muscle, 3% liver, 1% intestine, 0.5% heart, and 0.5%

kidney) were used in the nuggets formulation for the availability of tissue-dependent mitochondrial genes target.

7.3.4 RT-PCR Assay validation

For the validation of the developed assay, the actual values in admixed nuggets were compared with RT-PCR-determined values. A total of 15 model chicken nuggets with 0.01–100% of spiked dog meat were randomly selected. The percentage and DNA concentration of the known spiked samples were compared with the determined value as shown in table 7.3. A very good linearity (R^2 = 0.99) was observed when the actual values of the deliberately contaminated and RT-PCR predicted values were plotted (Figure 7.6). Application of the present model for nugget analysis showed excellent recovery rate of 87±28% to 112±19% for admixed analysis (%, w/w) and 89 ± 26% to 112± 19% for the detection of canine DNA (ng/µl) (Table 7.3).

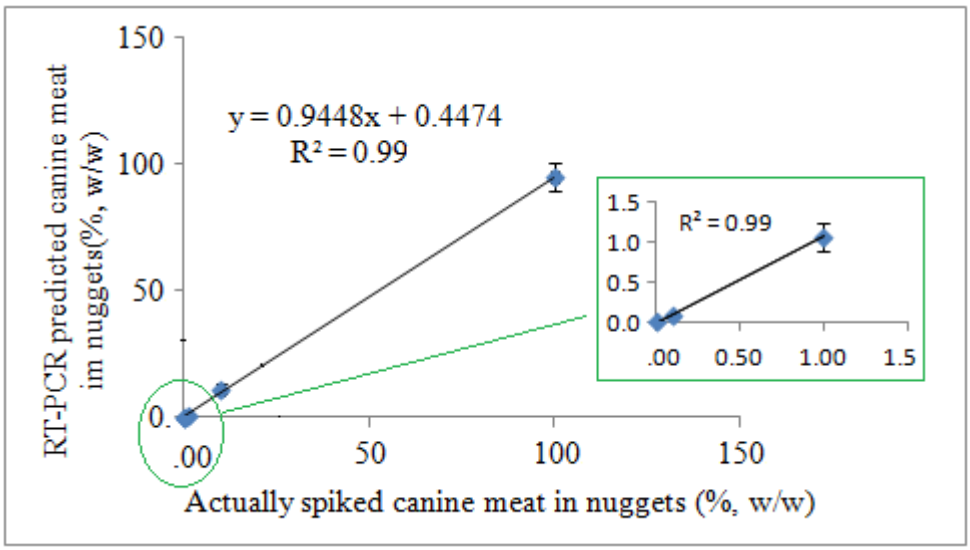

Figure 7.6 Relationship between actual and RT-PCR predicted canine meat admixed of 0.01 to 100 % (w/w) in chicken nugget. The inset is the relationship between actual and RT-PCR predicted canine meat % (w/w) in chicken nugget ranged from 0.01-1 % canine meat admixed.

Table 7.3 Validation of the real time PCR for canine species detection

Actual canine meat contamination		RT-PCR predicted canine meat contamination			
Admixed, %, w/w	Concentration ng/µl	Admixed %, w/w	Recovery, w/w (%)	Concentration ng/µl	Recovery, ng/µl (%)
100	20	94.79±8.39	94.56±8	19±1.712	95±9
10	2	11.29±1.95	112± 19	2.24±0.389	112±19
1	0.2	1.07 ± 0.18	107±18	0.20±0.028	101±14
0.10	0.02	0.10± 0.03	87 ± 28	0.02±0.005	89±26
0.01	0.002	0.01±.000	108±21	0.002±.0004	108±21

7.3.5 Commercial chicken nugget analysis

The major objective of this study was to test the potential of RT-PCR based assay for quantitative analysis of dog meat and its application in commercial sample testing. For achieving this goal, we had run the RT-PCR assay using 20 ng of DNA extracted from different nugget samples collected from three different outlets across Malaysia on three different days. Evaluation of different chicken nugget samples showed no amplification spectrum for the canine specific system (Table 7.4). However, the positive control of 0.01% deliberately canine meat contaminated samples showed canine specific amplification pattern at the range of 28.67±.44 to 29.21±.61 cycles. Amplification of endogenous control from the commercial sample between Ct values of 20.12±.21 to 21.06±.57 indicated the presence of good quality DNA in all samples. Thus, the absence of the canine DNA in commercial samples was proven by the non-amplification pattern of the DNAs from non-spiked nuggets within 40 cycles of RT-PCR reaction.

Table 7.4 Commercial nugget sample analysis

Chicken nuggets	Mean Ct Canine specific PCR system			Mean Ct Endogenous control			No of positive replicate
	Day1	Day2	Day3	Day1	Day2	Day3	
Canine meat spiked	28.67±.44[a]	29.21±.61[a]	29.20±.06 [a]	20.21±.23[a]	20.20±.63[a]	20.12±.21[a]	3/3
A	40[a]	40[a]	40[a]	20.44±.25[a]	20.36±.5 [a]	20.19±.29 [a]	3/3
B	40[a]	40[a]	40[a]	20.96±.35[a]	20.59±.29 [ab]	20.61±.52[a]	3/3
C	40[a]	40[a]	40[a]	20.83±.54[a]	20.69±.43 [b]	21.06±.57 [a]	3/3

Means with the same letter within the same column are not significantly different at 5% probability level.

7.4 Conclusion

A real-time PCR assay to determine canine meats in chicken nugget formulations was developed. The assay considered all the potential factors such as specificity, processing conditions, ingredients and meat tissue composition to validate the proposed model. The model experiment with calibration and validation sets showed no cross-species detection and strong correlation (R2.99) between the actual and predicted values. A high PCR efficiency of 99.7% and detection limit of 0.01% of canine DNA in chicken nuggets were obtained. Finally, analysis of 27 commercial chicken nuggets from Malaysian outlets revealed no canine adulteration of chicken nuggets in Malaysia.

CHAPTER 8
CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

Before making any food purchasing decision, we need clear and accurate information about the manufacturing ingredients of the food products. It will save guard our religious belief (e.g. vegetarianism, preference for organic products, absence of pork for Jews and Muslims) and will maintain our sound health (protect us from potential allergens and food born disease). Although techniques available for pig, horse and donkey meat detection, canine species and its adulteration detection remained as a less discussed issue. This dissertation is a comprehensive report on method developments for the determination of canine meats in processed meat products.

We developed here very short-amplicon length based PCR assays targeting a 100-bp site of cytb gene of canine mitochondrial DNA. The assays were tested for specificity, stability and sensitivity under raw, admixed various food processing conditions. Thus, we have optimized the assays under complex matrices and validated it by screening enough replicates of commercial meat products, namely frankfurter, meatball, kabsa, curry, burger and nuggets.

Extractions of DNAs from different raw and treated meat samples were performed using a commercial kit. However, to obtain a good quality and sufficient amount of DNA from the admixed, model or commercial food matrices the sample size was increased (25 mg > 100 mg-1gm) with subsequent purification steps. The cheap CTAB method allowed us to extract DNA from increased sample volume with minimize cost. Furthermore as ready to eat or commercial meat products are composed of complex DNA matrices with additional feed additives and or enzyme inhibitors. Therefore, we

have used additional phenol free purification kits and obtained a better quality DNA for PCR assay. The purity of the DNA samples was 1.90–2.0 at (A260/A280).

Species specific PCR is a simple and user friendly authentication tool for routine analysis of raw or processed foods. Hence, at first we have developed a canine specific PCR assay for the detection of canine biomaterials in process foods and validated its performance in frankfurter analysis. The specificity of the assay was tested against dog and 20 other common animal, fish and plant species which authenticated its specificity. The stability and sensitivity of the assay were verified under different thermal processing conditions under pure and complex matrices. The assay was found stable enough for the amplification of canine DNA either from raw or treated samples. Finally, three commercial brands of chicken and beef frankfurters were tested in triplicates and canine specific PCR products were obtained only from deliberately spiked samples. The detection limit of the assay for canine DNA materials was 0.1% (0.02 ng DNA) in a typical frankfurter formulation.

We further optimized and validated the assay for the screening of dog meat adulteration in meatball formulation. It selectively amplified the 100-bp region of canine mt-cytb gene from raw, processed and mixed samples. The specificity of the assay was tested against 11 animals and 3 plants species, commonly found in meatball formulation. The stability of the assay was proven under differentially treated meatballs samples such as raw, boiled and extensively autoclaved condition that may breakdown target DNA. A blind test using ready to eat chicken and beef meatballs showed that the assay can repeatedly detect 0.2% canine tissues under complex matrices using 0.2 ng/20 ng of total DNA extracted from different treated meatballs. Thus, the specificity, stability and sensitivity of this assay suggested that, it can be used in food industries and halal authentication laboratories for the authentication of canine derivatives in meatball formulations.

The well developed short amplicon based canine specific PCR assay was further tested in kabsa and analysis of curry which are popular dishes in Arabian Peninsula and Indian subcontinent. The discrimination of canine origin materials was performed through the use of the pair of cytb based canine specific primers. Here, the canine specificity of the primers was tested with total fourteen ten potential animals including dog and other plant species DNA which are used in kabsa and curry formulation and the was proven for its desired specificity. Furthermore, the targeted fragment demonstrated high stability under different food processing conditions such as boiling, autoclaving; steam cooking, oven heating and salting and sensitivity under complex pool of admixed samples. Finally, the assay repeatedly detected 0.1% (0.02 ng) canine DNA target in deliberately contaminated kabsa and curry. However, the analysis of six different commercial kabsa and curry samples collected from different Malaysian outlets did not reveal any dog meat contamination. Thus the potential application of this assay for testing Arabian and Indian cuisine may found across the globe including Arabian Peninsula, Indian subcontinent and Malaysia which is a popular tourist destination.

The authenticity of the developed PCR assay was confirmed through restriction digestion and RFLP analysis. Due to narrow differences in fragment-lengths, a micro-fluidic based automated electrophoresis system incorporated onto to Bioanalyzer chip was used for fragment separation and distinctive restriction patterns were obtained. We optimized and validated the RFLP assay for the analysis of processed foods such as commercial burgers. Both gel-image and electropherograms authenticated the PCR product before (100 bp) and after digestion (51-, 30- and 19-bp). The assay successfully detected 0.0001 ng canine DNA under pure state and 0.01% (w/w) canine meat (0.002 ng canine DNA) spiked with chicken and beef burger formulations. Screening of eight commercial burgers across Malaysia did not reveal any canine adulterations.

Recent meat-forgery scandals and on growing zoonotic threats of various meat-borne diseases have made it a necessary to develop techniques for the detection and quantification of canine species in meat products to maintain health, religious faith, fair-trade economy. Therefore we have developed the short-amplicon based TaqMan probe real-time polymerase chain reaction (RT-PCR) assay for the detection and quantitative of canine meats in processed foods such as chicken nugget, which is a very popular food product in most parts of the world including Malaysia. The assay targeted the 100-bp fragment of canine cytb gene using a pair of canine specific primers and TaqMan probe. Specificity analysis against 10 different animals and plants species demonstrated a threshold cycles (Ct) of 16.13±.14 to 16.25±.23 for canine and negative results for the others in a 40-cycle reaction. The assay was tested for the quantification of up to 0.01% canine meat in deliberately spiked chicken nuggets with 99.7% PCR efficiency and 0.99 correlation coefficient. The analysis of the actual and RT-PCR predicted values revealed a high recovery rate (87 ± 28% to 112± 19%) with linear regression close to unity (R^2 0.99). Finally, total 27 samples of three halal branded commercial chicken nuggets collected in triplicates on three different days from different Malaysian outlets were screened for canine adulteration and negative detection was demonstrated.

The developed qualitative and quantitative canine biomarker based assay platforms showed the potential for applications in food industries and regulatory bodies for determine food adulteration and Halal authentication. Brief summary of the present work according to the objectives are given below:

1. Canine Specific Biomarker Development

The developed canine biomarker was tested for specificity against 26 different species of terrestrial and aquatic origins including meat providing animals, fish and plant species commonly used in different food formulations to demonstrate its

potentiality to detect canine material with 100 % accuracy. In-silico analysis reflected the presence of minimum 5 mismatches in the primer binding site, eliminating the probability of any non-target DNA amplification. Repeated cross-PCR runs also confirmed that the developed primers were solely specific for the canine species.

2. Validity of the Biomarker

The canine specific 100 bp biomarker was primarily validated for stability under raw, autoclaved and different food processing steps and was able to detect canine DNA from all samples. The biomarker was further validated for its sensitivity under different admix and four popular commercial food matrices, namely frankfurter, meatball, Kabsa and curry using conventional PCR assay. It detected up to 0.1% of canine meat under all matrices. The biomarker was also tested using Lab-on-a-chip based assay in commercial burger formulation. The highly sensitive lab-on a chip based assay was able to detect up to 0.01% of canine meat. Finally, the validity analysis using RFLP method showed the potentiality of the assay to amplify only canine DNA with three specific fragments of 19 bp, 30 bp and 51 bp size. Different frankfurter, meatball and burger analysis from total 21 different brands and Kabsa, curry from 8 different outlets of Malaysia showed no canine meat positive samples, demonstrating the reliability of the Halal labeled food items in Malaysia.

3. Quantitative canine specific assay development

A 100 bp amplicon based qPCR assay was developed for quantitative detection of canine DNA based on 24 bp TaqMan Probe and canine specific primer. The specificity of the assay was tested in commonly used 10 different species DNA and showed its reliability to detect only the canine species. Furthermore the validity analysis of the assay under commercial chicken nugget formulation showed the Ct values of 16.34±0.28 (100%) to 29.22±0.18 (0.01%) under different percentages of dog meat

spiked chicken nuggets. The developed qPCR assay has 99.7% PCR efficiency with a recovery rate of 87 ± 28% to 112± 19%. This is the first time report of a canine specific qPCR assay which was validated for commercial food sample such as chicken nugget. No samples from 3 different brands (27 samples) of chicken nuggets from different Malaysian out let was found positive for canine meat.

The present research has due importance and proven for its application in different meat products analysis to determine fraudulent admixing of dog meat. We have tested the assay performance in economically important food products and also screened food products from different outlets of Malaysia.

8.2 Recommendation for future work

The developed food authentication assays in this study are promising tool to detect and quantify specific DNA target from contaminated and highly degraded samples. Detection of specific DNA target is a very useful technique to distinguish permissible and non-permissible food materials to safeguard human health, religious view and to secure fare trade. The present methodology can be used for the detection of cheap fish species adulteration in minced cod or Alaska pollock. Previous literature supported the development the cytb based canine specific PCR assays using short length DNA target. However, due to time constrain we did not went through side by side real run analysis of our and described PCR assays. Future work based on this described short length DNA target along with new target such as different gene on same length or same gene on different length may aid for better understanding of gene nature and effect of different primers for species authentication.

Human food products are composed of different components and most of the time with multiple species background. Hence for authentication of food products multiple species targets may an alternative to the present assays. The present work has the drawback of specific target which is limited to the detection and quantification of a single species. Future work can explore the application of multiple species target which can detect and quantify different species DNA from a complex pool of food matrices. A multiplex PCR assay platform can be designed by incorporating specific primer pairs from different potential Haram (not allowed) meat species such as pork, cat, rat, horse etc in a single assay run. A species detection platform as “Multiplex Halal detection Kit”, can serve as an alternative to “Singleplex” assay to serve food authentication from different species background. This future assay technique may save time and use of expensive consumables.

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Appendix A

Front pages of the publication

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Canine-Specific PCR Assay Targeting Cytochrome b Gene for the Detection of Dog Meat Adulteration in Commercial Frankfurters

Md. Eaqub Ali · Md. Mahfujur Rahman · Sharifah Bee Abd Hamid · Shuhaimi Mustafa · Subha Bhassu · Uda Hashim

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Abstract This report described a cytochrome b (cytb)-based polymerase chain reaction (PCR) assay for the detection of canine tissues in commercial frankfurters. Discriminating detection of canine derivatives in processed food products has important application in halal authentication as well as in health, religions, and fare trades. The assay based on a pair of canine-specific primers that targeted a 100 bp region of canine mithochondrial-cytb gene which is present in multiple copies and highly conserved within the same species. The specificity of the assay was tested against dog and eight most common animal meat species as well as five plant species commonly found in frankfurter formulation. The stability and specificity of the assay were verified under different thermal processing conditions under pure and complex matrices. Three commercial brands of chicken and beef frankfurters were tested in triplicate, and specific PCR products were obtained only from deliberately contaminated formulations. The detection limit of the assay was 0.1 % (0.02 ng DNA) of canine meat spiked with other meats in a typical frankfurter formulation. Shorter amplicon length, superior stability, and higher sensitivity of the assay suggested its potential application in the screening of canine-origin biomaterials in processed food products.

Keywords Canine-origin biomaterials · Halal authentication · Commercial frankfurters

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Introduction

The authentication of meat species is an increasingly concern and a vital part to ensure quality foods in compliance with health, religions, and fair prices (Ali et al. 2012a, b; Rohman et al. 2011). Furthermore, the newly emerging "zoonotic threat which is an animal origin infectious disease capable of being transmitted to human and other animals" has tremendously added drive toward meat species identification and meat quality authentication (Karabasanavar et al. 2011). According to European law, food manufacturers must declare and clearly label ingredients used in the preparation of both raw and finished foods (Commission 2002). Including Malaysia, many countries in the world either already have or are being having regulatory bodies to ensure proper labelling as well as Halal status of the processed foods (Ali et al. 2012a, b, c; Musa and Jalil 2012).

"Halal" logo on food products is trusted by 1.8 billion Muslims of the globe, and it signifies that the products are prepared following the Shariah law of Islam for hygienic foods, and Muslims have no religious obstructions to consume those

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Polymerase chain reaction assay targeting cytochrome b gene for the detection of dog meat adulteration in meatball formulation

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ABSTRACT

A polymerase chain reaction (PCR) assay for the assessment of dog meat adulteration in meatballs was developed. The assay selectively amplified a 100-bp region of canine mitochondrial cytochrome b gene from pure, raw, processed and mixed backgrounds. The specificity of the assay was tested against 11 animals and 3 plants species, commonly available for meatball formulation. The stability of the assay was proven under extensively autoclaving conditions that breakdown target DNA. A blind test from ready to eat chicken and beef meatballs showed that the assay can repeatedly detect 0.2% canine meat tissues under complex matrices using 0.04 ng of dog DNA extracted from differentially treated meatballs. The simplicity, stability and sensitivity of the assay suggested that it could be used in halal food industry for the authentication of canine derivatives in processed foods.

1. Introduction

The prospects for Halal meats and meats products are rapidly expanding (Ali, Hashim, Dhahi, Mustafa, Man, et al., 2012). Currently, halal food consuming population has reached to 1.8 billion and the turnover of halal market has exceeded USD 661 billion (Ali, Kashif, et al., 2012). Several factors including increasing workloads are pushing potential halal consumers to spend more time in workplaces, leaving no time for self-cooking. Thus a growing number of people are increasingly being forced to eat readymade foods such as burger, pizza, hot dogs, meatball, soups and so on (Ali, Kashif, et al., 2012). Due to the specialized preparation of halal meats, the prices for halal brands are higher, especially in those countries where Muslims are the minority. Thus the fraudulent labelling of "halal" brands is prevalent (Ali, Hashim, Mustafa & Che Man, 2012). To cope up with the situation demands and business requirements, many countries including Malaysia, Indonesia, Thailand, Singapore, China, Brazil, Australia and Newzealand are having regulatory bodies to protect the sanctity of Halal food markets (Ali, Kashif, et al., 2012). Thus the food manufacturers, marketers and regulators need innovative, easily performable and improved authentication techniques for the verification of halal brands.

Meatballs made up with comminute meats are very popular throughout the world including Malaysia, Indonesia, China, Vietnam, India, the USA and the Europe (Ali, Hashim, Mustafa, Che Man, Dhahi, et al., 2012; Rohman, Sismindari, Erwanto, & Che Man, 2011). Dog meat is a potential adulterant in halal foods since stray dogs are available in many countries without any offered prices. Reports have been made for the consumption of dog meats in certain countries such as Vietnam, South Korea and China (Bartlett & Clifton, 2003; Podberscek, 2009). Foreign workers, especially from Myanmar and Vietnam origins, are reported to consume stray dog meat in Malaysia. However, no survey is made to verify the mixing of dog meats in commercial meat products across Malaysia or elsewhere in the world. The mixing of dog origin materials in food is a serious issue in many religions including Islam and Buddhism (Khattak et al., 2011; Mahanarongchai & Marranca, 2007).

Several methods such as SYBR green real-time PCR (Farrokhi & Jafari Joozani, 2011), molecular beacon real-time PCR (Yusop, Mustafa, Che Man, Omar, & Mokhtar, 2012), TaqMan probe real-time PCR (Ali, Hashim, Dhahi, et al., 2012), electronic nose coupled with gas chromatography–mass spectrometry (Nurjuliana, Che Man, Mat Hashim, & Mohamed, 2011), Fourier transform infrared spectroscopy (Rohman et al., 2011), enzyme-linked immunosorbant assay (Asensio, González, García, & Martin, 2008), PCR-RFLP (Ali, Hashim, Mustafa, & Che Man, 2012) and nanoparticle sensors coupled with optical or fluorescence spectroscopy (Ali et al., 2011) have been proposed for the authentication of meat species. Although cumbersome to some extent, the conventional species specific PCR assay is an easily affordable and reliable method for the routine analysis of animal meat products in food industry (Arslan, Ilhak, & Calicioglu, 2006; Matsunaga et al., 1999). For dog

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Lab-on-a-Chip PCR-RFLP Assay for the Detection of Canine DNA in Burger Formulations

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Received: 7 October 2014 / Accepted: 7 January 2015

Abstract Canine species detection in foods is important in the perspectives of health, religions, and fare-trade food business. This study describes a very short-amplicon length Polymerase Chain Reaction (PCR)-Restriction Fragment Length Polymorphism (RFLP) assay with lab-on-a-chip detection platform for the authentication of canine DNA in processed foods. A 100-bp fragment of canine mitochondrial Cytochrome b (cytb) gene was selected and amplified using a pair of canine-specific primers. The amplified PCR products were validated by RFLP analysis using lab-on-a-chip microfluidic bioanalyzer kit. Both gel-image and electropherograms authenticated the canine-specific PCR products before (100 bp) and after restriction digestion (51, 30, and 19 bp). The assay successfully detected 0.0001-ng canine DNA under pure state and 0.01 % (*w*/*w*) canine meat spiked in chicken and beef burger formulations. Screening of eight commercial burgers across Malaysia did not reveal any canine adulteration. We believe the assay would find potential applications in food industries, Halal food regulatory bodies and animal right protection authorities across the globe.

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Keywords Burger formulation · Lab-on-a-chip bioanalyzer kit · Microfluidic capillary electrophoresis · PCR-RFLP

Introduction

It is a long-term envision of human civilization that food will be compliant with health, religions, culture, and age (Ali et al. 2014). While elders are concerned of healthy foods, younger often run after taste, appearance, and availability (Nam et al. 2010). In contrast to the consumer ages, religions often play a key role in controlling the consumption, preparation, processing, and purchasing of foods (Bonne and Verbeke 2008; Nam et al. 2010). Most religions have food taboos, for example, pork is not allowed to be consumed for the followers of Islam and Judaism (Bonne and Verbeke 2008). In Islam, meats of the ritually slaughtered animals with split hoof such as sheep, cattle, buffalo, and goat are allowed, but those of the carnivores with sharp teeth such as dog and cat are forbidden to be consumed (Khattak et al. 2011).

The term "Halal" is an Arabic word which defines the permitted things for the Muslims by the Islamic law drawn from the divine book of Quran and the compilation of Prophet Muhammad (Hadith). The "Halal" status of processed foods cannot be verified by consumers using organoleptic test or even after consumption since the processing treatments significantly modify the organophysical biomarkers, making the physical identification extremely difficult (Bonne and Verbeke 2008). Therefore, "Halal" logo on food products are trusted by the consumers, and it authenticates the halal status of the food and its ingredients. Due to huge demand (US$700 billion annually) and higher price of Halal foods, fraudulent labelling of halal brands are frequently taking place (Ali et al.

Appendix B

Conferences

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Identification of short-length oligonucleotides biomarker for canine species detection using mitochondrial cytochrome b gene

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ABSTRACT

Introduction: Stray dogs are still available in certain countries without any offered price and made it as a potential source for adulteration with costly meats for more benefit. Furthermore, human forensic evidences from crime scenes were often integrated with biomaterial of canine origin. Most of the DNA based assay for canine species detection used longer amplicon size (>150 bp) which are not suitable for highly degraded food or forensic sample analysis. Therefore, in this study for development of short length canine specific biomarker, mitochondrial cytochrome b (cytb) gene was targeted using simple PCR assay.

Objective: Detection of canine species using short length DNA biomarker targeting cytb gene.

Methods: The assay targeted a 100-bp fragment of cytochrome b gene using a pair of canine specific primers. The primers specificity were tested under Insilco, as well as in real PCR assay using dog and eight other species DNAs. The consensus 100 bp canine specific site along with cytb sequences of 14 species including dog and human were used for analysis of pair wise distances, construct dendogram and primers mismatch calculation. The stability of the biomarker was tested under commonly used cooking condition and extensive autoclaving state which was known for degradation of target DNA. The sensitivity of the assay was tested using binary admixture composed of dog and most consumed chicken DNA pool.

Results & Discussion: The biomarker was 100% canine specific and successfully amplified 100 bp region of canine cytb gene specific target. It was highly stable and sensitive enough to detect as low as 0.1% (0.02 ng) of canine specific target from admixed DNAs.

Conclusion: The primers provided the shortest DNA biomarker for canine species detection. The shortest amplicon length, high stability and sensitivity offered its potentiality for canine biomaterials determination from food as well as from degraded samples.

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May 8,2014

Dear Author(s),

On behalf of the INNOVAFOOD2014 organizing committee, we would like to thank you for submitting your abstract to the International Conference on Food Innovation: INNOVAFOOD2014. We are pleased to confirm that your abstract has been accepted for the conference:

Author : Md. Mahfujur Rahman, Md. Eaqub Ali, Sharifah Bee Abd Hamid, Shuhaimi Mustafa, Subha Bhassu, Raifana Rashid (**ORAL**)

Title : Canine Specific Polymerase Chain Reaction Assay Targeting Cytochrome B Gene for Species Authentication

We kindly invite you to submit your full paper via the conference email: innovafood2014@usm.my. Please take note that the due date for submission is **June 16, 2014.** We would encourage the participants to register at the earliest possible time as it will facilitate the final scheduling. The instruction for registration and paper submission are available on our website: www.innovafood2014.usm.my.

We are looking forward to receive your full paper and should there be any enquiry, please do not hesitate to contact us.

Thank you.

Your sincerely,

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Certificate of Appreciation

MD. MAHFUJUR RAHMAN

(PARTICIPANT)

This is acknowledgement to your contribution to the Joint International Conference on Nanoscience, Engineering and Management (BOND21) held at Bayview Beach Resort, Penang, Malaysia on 19th – 21st August 2013.

Prof Dr Uda Hashim
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This is certify that :

Md. Mahfujur Rahman

has participated and presented the paper entitled:

Gold Nanoparticles - An Enhanced DNA Sensing Tools Using Surface Enhance Raman Scattering

Md. Eaqub Ali, Sharifah Bee Abd Hamid

at the **Malaysia-Japan International Conference on Nanoscience, Nanotechnology and Nanoengineering 2014 (NANO-SciTech 2014 & IC-NET 2014)**

held in **UiTM Shah Alam**, Selangor,

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